Title Studies on Limiting Factors Relating to the Cryopreservation of Fish Embryos

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STUDIES ON LIMITING FACTORS RELATING TO THE CRYOPRESERVATION OF FISH EMBRYOS

by

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To

My Mother

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ABSTRACT

Cryopreservation of fish embryos has proven to be a difficult problem in cryobiology. Three main difficulties have been identified or suspected: 1) embryo membrane permeability barriers to cryoprotectants and water; 2) high chilling sensitivity of the embryos; and 3) the two-compartment nature of the embryos with a large yolk. Using the zebrafish embryo as a model system, these limiting factors and possible approaches to overcoming them were investigated with a view to developing an effective procedure for fish embryo cryopreservation.

Compared to previous studies, vitrification of zebrafish embryos on gold electron microscope grids using methanol as the cryoprotectant resulted in improved morphological survival, being ~ 50% for early stage (1-cell and 64-cell) and ~ 80% for late stage (50%-epiboly, 6-somite and prim-6) embryos, but no embryo showed viability. Poor cryoprotectant permeation and embryo dehydration, and consequently intraembryonic ice formation, remained as the main problem for vitrification. Embryo chilling sensitivity studies suggested that later stage zebrafish embryos were sensitive to cold shock injury arising from rapid cooling followed by being held for an extended exposure period (1 h) at 0 or -5°C. Studies on embryo developmental arrest by anoxia showed that chilling injury in zebrafish embryos was probably not associated with their high development rate. However, the chilling sensitivity of zebrafish embryos was found to be related to the amount of yolk present. Yolk-reduced embryos at prim-6 and high-pec stages became less sensitive to chilling at 0°C. Differential scanning calorimetry studies on the depression of intraembyonic nucleation temperatures by cryoprotectants revealed that multi-punctured embryos at 6-somite and prim-6 stages became significantly more permeable to methanol and propylene glycol when compared with their non-punctured controls. Puncturing of the yolk-sac of fish embryos to reduce yolk content, and the increased permeability to cryoprotectants that this promotes, may offer a new approach to surmounting the difficulties confronting the cryopreservation of fish embryos.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
CPAs	cryoprotective agents
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
EM	embryo medium
FTIR	Fourier transform infrared spectroscopy
IIF	intracellular ice formation
LAVCs	lowest apparent vitrification concentrations
LN_2	liquid nitrogen
NADH	nicotinamide adenine dinucleotide
PG	propylene glycol
SEM	standard error of the mean
YSL	yolk syncytial layer

CHAPTER 1 INTRODUCTION

1.1 Aim of the research and significance

Whilst considerable progress has been made over the last two decades in the cryopreservation of mammalian embryos, the successful cryopreservation of fish embryos has remained elusive. The aim of the present work was to investigate the limiting factors relating to the cryopreservation of fish embryos and to overcome them with a view to developing an effective cryopreservation protocol.

The development of effective cryopreservation procedures for fish embryos has great potential for facilitating the conservation of endangered populations of fish, management of wild stocks in fish farming and ecotoxicological testing on early life stages. Based on reports of the red data books of the International Union for Conservation of Nature and National Resources, more than 65% of the European fish species are threatened (Kirchhofer, 1996) and extremely endangered species may be extinct before recolonisation is possible or their genetic variability so reduced that reconstruction of stable population is impossible (Gilpin and Soule, 1986). Fish embryo cryopreservation would: (1) maintain species diversity; (2) prevent species extinction by producing a reservoir of 'insurance' populations; and (3) allow time to rehabilitate the native environment to support species re-introduction.

In fish farming and fish culture, the successful cryopreservation of fish embryos would offer new commercial possibilities by: (1) allowing the production of fish throughout the year; (2) reducing the size and production cost of aquaculture facilities; (3) allowing the maintenance of a large gene pool and reducing inbreeding, while minimising the amount of space required to hold living animals; (4) maintaining a constant supply of animals; (5) reducing the impact of aquaculture sites on wild populations; and (6) facilitating global, regional and institutional transport of genetic material.

Fish embryos or eggs are of particular interest for ecotoxicological testing because of their environmental relevance and sensitivity. Early life stage tests have been favoured to replace acute fish tests which are less acceptable to society. National and international environmental protection laws require toxicity testing of dangerous substances and sewage

samples, in order to obtain data on toxicity and for biological monitoring. For enforcement it is important to analyse field samples immediately. Successful cryopreservation of embryos or eggs would guarantee time-independent availability of qualitatively homogenous biological material for fish-egg tests.

1.2 Principles of cryobiology

Cells and tissues are usually damaged or even killed following exposure to low temperatures, and yet paradoxically under low temperatures, cells, tissues and even organisms can be sustained for extended periods such as months, years or even centuries because low temperatures slow or stop biochemical reactions. Although the consequences of the exposure of living cells to lowered temperatures are diverse and paradoxical, there are fundamental underlying mechanisms that determine how all biological systems respond to the lowering of temperature and the solidification of liquid water.

Two notable damages can usually be classified after cells and tissues have been exposed to lowered temperatures: chilling injury, which is used to refer to the damage following exposure to low temperature without freezing, and freezing damage.

1.2.1 Chilling injury

Many types of cells and tissues are damaged when cooled to temperatures close to or below 0°C without freezing. The term "*chilling injury*" was originally used in the botanical world as early as the eighteenth century to describe the phenomenon that plants subjected to chilling temperatures above the freezing point of water were often damaged irreversibly (Levitt, 1980). In 1934, Milanov (cited in Watson and Morris, 1987) first coined "*temperature shock*" to describe the irreversible damage to mammalian sperm that occurs when these cells are cooled rapidly (faster than a few degrees per minutes) below body temperature. It has been recognised that temperature shock in sperm and chilling injury in plant cells are probably related mechanistically. A more common term "*cold shock*" is used to describe both phenomena, which is expressed quickly upon reduction in temperature and is dependent on cooling rate (Morris and Watson, 1984). Another category of chilling injury which is independent of the rate of cooling and usually manifested after extended periods of

exposure to low temperatures is termed *"indirect chilling injury"* (Morris and Watson, 1984). Studies with mammalian embryos show that cooling rate has little influence on overall embryo survival after chilling, but chilling injury may develop very rapidly (Plante *et al.*, 1993; Martino *et al.*, 1995). In some cases, it seems difficult to classify the effects of cooling as due exclusively to *cold shock* or to *indirect chilling injury*. These two categories are probably components of the same phenomenon. Differences in the response of different types of cells may be quantitative rather than qualitative (Watson and Morris, 1987).

1.2.1.1 Cold shock

The essential features of cold shock have been outlined as follows (Morris, 1987):

(a) all cell types may be considered sensitive to cold shock provided that they are cooled rapidly enough to a sufficiently low temperature;
(b) cellular viability is dependent upon the rate of cooling with more injury observed following "rapid" rather than "slow" cooling;
(c) cold shock injury is almost independent of the rate of warming, when this does not significantly increase the length of exposure to low temperature;
(d) injury is increased as the period of isothermal incubation at the reduced temperature is extended;
(e) loss of membrane permeability occurs following rapid cooling, and in some instances, may be reversed upon rewarming;
(f) the response of any cell-type may be modified by the culture conditions before cooling or by the addition of specific compounds.

It is generally agreed that it is the thermotropic behavior of membrane lipids which is the factor determining cold shock injury (Morris, 1987). The correlation between cold shock behavior and lipid composition of the sperm from various species suggests that this form of injury is associated with thermotropic phase transitions in sperm membrane lipids (Watson, 1981; Watson and Morris, 1987). Freeze-fracture studies have demonstrated that cooling the sperm of sheep from 30 to 5°C (Holt and North, 1984), or those of cattle or pig from 38 to 0°C (De Leeuw *et al.*, 1990) causes morphological membrane changes consistent with a lipid phase transition in this temperature range. Fluorescence polarisation studies with plasma membrane vesicles prepared from sheep (Holt and North, 1986) and pig (Canvin and

Buhr, 1989) sperm have also shown changes in apparent viscosity of the membrane in the temperature range below which cold shock occurs. Recently, using Fourier transformation infrared spectroscopy (FTIR), lipid phase transitions in the cell membranes have further been confirmed to be responsible for cold shock injury in sperm (Drobnis *et al.*, 1993), bovine oocytes (Arav *et al.*, 1996), and human platelets (Crowe *et al.*, 1999). The lipid phase transitions from the liquid crystalline to the gel phase is accompanied by leakage of solutes across membranes (Jain, 1983; Waston and Morris, 1987). Phase separation which is associated with the lipid phase transitions is believed to damage membranes by several mechanisms. Formation of "packing faults" between lipid domains of different phases may disrupt membrane permeability (Pringle and Chapman, 1981; Jain, 1983), and the occurrence of concentrated lipids that tend to form non-bilayer phases (Quinn, 1985) as well as aggregation of mobile, intrinsic proteins within the remaining liquid crystalline domains (Pringle and Chapman, 1981; Quinn, 1985). Rapid rates of cooling cause membrane phase separation which results in more defects than slow cooling. Hence rapid chilling would be likely to produce leaky membranes and cause severe damage to cells.

McGrath (1987) proposed a different mechanism of cold shock named the thermoelastic stress theory, which describes the dynamic tension which results within a fluid membrane vesicle as the temperature is reduced rapidly. This theory is based on the fact that reduced temperature will result in membranes attempting to thermally contract laterally around an essentially incompressible aqueous interior. The contraction will cause the efflux of cell water, which will concentrate the cytoplasmic solution and reduce its chemical potential, thus setting up an osmotic force to drive water into the cell. The result will be a finite stress or tension on the membrane. Sufficient tension to cause damage to liposomes will develop for temperature decreases of 10 - 20°C, larger temperature decreases will create greater tensions. The tension developed in membranes surrounding small vesicles with diameters < 0.2 μm will also depend on the water permeability of the vesicles and the cooling rate, because the water permeability will set a limit on the rate at which the contraction-induced water efflux can occur relative to that required for equilibrium as the cells are cooled at various rates. The faster cooling rate will produce increased tension for a given temperature reduction. Therefore cold shock will be observed in the case of rapid cooling.

1.2.1.2 Indirect chilling injury

Indirect chilling injury is usually evident following a relatively long exposure period at the reduced temperatures, and it is independent of the rate of cooling. But in mammalian oocytes or embryos and some early stage insect embryos, indirect chilling injury may manifest rapidly (within minutes) (Leibo *et al.*, 1996, Mazur *et al.*, 1992). The structure and activity of both proteins and lipids could be modified following a reduction in temperature (Morris and Clarke, 1987).

A potential sequence of events may occur in cellular membranes in response to a reduction in temperature (Fig. 1.1). The initial effect of a temperature reduction will be an increase in membrane viscosity. A phase separation may then occur, depending on the cell-type and the extent of temperature reduction. Both events will have immediate effects on membrane function. Although many cell-types can offset phase changes by modifying the lipid composition of their membranes, these compensations are rarely perfect. Cells will usually be trapped in a state of permanent phase change or transition after an extended period of exposure to low temperatures. Following phase transition, many biological properties of the membrane are altered, and this leads to the alteration of the activities of membrane proteins and membrane-associated enzymes (Cossins, 1983).

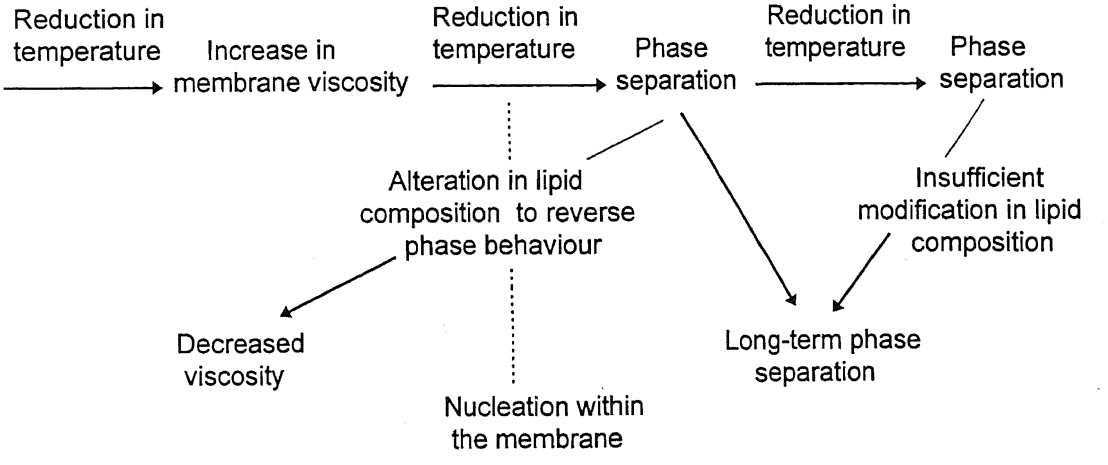


Fig. 1.1 Flow diagram of membrane events during a reduction in temperature, indicating potential long-term responses (from Morris and Clarke, 1987).

Low temperature may affect the structure and function of proteins, for example by decreasing the rate of enzyme activity and the denaturation of proteins (Jaenicke, 1981). Even the simplest enzyme-catalysed reactions involve several coupled steps, each of which is likely to have a different activation energy. A reduction in temperature will be expected to affect different enzyme reaction rates to a different extent. The effects of these alterations in enzyme activity on the series of linked reactions are complex and metabolic pathways are likely to become uncoupled. The disorder of metabolic and enzymatic processes can be especially detrimental in fast developing embryos like *Drosophila* and such injury will increase rapidly at lower temperatures because the loss of co-ordination is increased with decreasing temperature (Mazur *et al.*, 1992). In addition to these effects, reduced temperatures may also affect the cytoskeleton system, for example, the low temperature depolymerisation of microtubules (Behnke and Forer, 1967; Weber *et al.*, 1975; Magistrini and Szollosi, 1980), which could result in the irreversible disruption of cellular processes like cell division in oocytes (Magistrini and Szollosi, 1980; Martino *et al.*, 1995)

1.2.2 Freezing damage

Liquid water is essential to the structure and function of living cells, and solidification of water by freezing is usually destructive or even lethal to cells. The stresses to which cells are exposed during freezing will mainly result from the following three aspects (Grout and Morris, 1987):

(a) the mechanical effects of extracellular ice crystals at cell surfaces, especially in tissues with cellular interconnections
(b) alterations in the physical properties of solutions outside the cell, including the concentration of solutes which results from the nucleation of a proportion of extracellular water;
(c) intracellular freezing if it occurs.

Cellular responses to freezing are the direct result of physical laws and are not under any sort of biological control. The immediate responses of a cell, or organelle, are governed by physical and chemical properties of both the biological and physical systems involved, and they will tend to establish a new set of equilibrium conditions.

1.2.2.1 Physicochemical events during freezing

When the temperature is down to about -5°C, the cells and their surrounding medium remain unfrozen both because of supercooling and the depression of the freezing point by the protective solutes that are frequently present. Between about - 5°C and - 15°C, ice forms in the external medium either spontaneously or as a result of deliberate 'seeding' of ice, but the cell contents remain unfrozen and supercooled. One of the most fundamental consequences of the presence of ice in the external medium is the effect on the composition of the unfrozen fraction of the extracellular solution. Because solute concentration in the extracellular solution increases as the temperature decreases and the ice phase grows, a chemical potential imbalance between the biomaterial and the unfrozen external solution results. The supercooled water in the cells has, by definition, a higher chemical potential than that of water in the partly frozen solution outside the cell, and in response to this difference in potential, water flows out of the cell and freezes externally (Mazur, 1984; Toner, 1993). The subsequent physical events in the cell depend on the cooling rate.

If cooling is sufficiently slow, the cell is able to lose water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. If the cooling is too rapid, the rate at which the chemical potential of water in the extracellular solution decreases is much faster than the rate at which water can diffuse out of the cell and the end result in intracellular ice formation (IIF).

These qualitative statements could be described quantitatively. The first mathematical model of the kinetics of cell dehydration during freezing was produced by Mazur (1963). The model was later modified by Pitt (1992) and Toner (1993). These mathematical models permit one to calculate the extent of supercooling in the cell as a function of cooling rate, provided one knows or one can estimate the permeability of the cell to water, its activation energy, the osmoles of solute initially in the cell, and the ratio of the cell surface area to volume.

By definition, the water-ice phase transition in an aqueous solution is thermodynamically favoured only at temperatures below the equilibrium melting point. When ice forms in such a supercooled solution, solutes are rejected from the growing ice front, and the unfrozen fraction of solution becomes increasingly concentrated. The concentration of extracellular solutes in the residual unfrozen medium increases according to the relation

$$C = \Delta T / 1.86$$

where C is the external osmolity, Δ T is number of degrees below 0°C. The value 1.86 is the molal freezing-point depression constant for water. In partly frozen solution, C is independent both of the nature of the solutes and of their total concentration prior to freezing. The equilibrium osmolity of the unfrozen fraction is a function of temperature only.

From the above discussion, it is clear that cooling can impose either of the following two conditions of stress on cells. Firstly, during 'slow' cooling the major stresses are likely to result from extracellular freezing, whereas during 'rapid' cooling the stresses are likely to result from intracellular ice formation. This situation is recognised in the 'two factor' hypothesis of freezing injury which essentially ascribes cellular injury at sub-optimal cooling rates to damaging effects of prolonged exposure to hypertonic solutions ('solution effects') whilst at super-optimal rates of cooling the nucleation of intracellular ice results in cell death (Mazur *et al.*, 1972).

1.2.2.2 Extracellular freezing

Biological systems will be subjected to a series of stresses arising from the formation of the extracellular ice. There have been several theories suggested to explain the cause of the damage although the exact mechanisms of cell damage during extracellular freeing have not been fully understood.

Increase in concentration of extracelluar electrolytes Lovelock (1953, 1953a, 1954) suggested, on the basis of freezing red blood cells, that much of the freezing damage could be accounted for by the increased salt concentration, particularly electrolytes in the unfrozen aqueous fraction, which in turn leads to an increase in the concentration of intracellular electrolytes. There was evidence that hypertonic salt solutions caused denaturation of lipoproteins, and that this process could induce haemolysis in red blood cells (Lovelock, 1957). At higher electrolyte levels denaturation of enzyme activity may occur by modification of the weak molecular interactions responsible for maintaining the native protein structure (Jaenicke, 1981).

Reduction in cell volume Meryman and his associates proposed the 'minimum cell volume theory' to account for cellular damage associated with freezing and thawing (Meryman *et al.*, 1977; Williams and Shaw, 1980; Clegg *et al.*, 1982). Like Lovelock's theory, it proposed that high salt concentration during freezing was important, but differed in suggesting that salt concentration was indirectly responsible for damage, with high extracellular osmolarity causing cells to be desiccated beyond their limit, that is shrunken below their "minimum cell volume" and thus the cells were destroyed.

Mechanical effects of ice Nei (1967) postulated that cells are damaged by the mechanical interaction between the growing ice phase and cells sequestered between ice crystals and this mechanical damage is a significant cause of hemolysis in erythrocytes (Nei and Tanno, 1968). Later, it was shown by Mazur *et al.* (1981, 1983, 1989) that during slow cooling, survival of human erythrocytes, at any particular temperature, is more dependent on the magnitude of the unfrozen fraction than on the salt concentration in the extracellular solution. They concluded that the dominant mechanism of damage during freezing at a slow cooling rate is of a rheological nature , related to the interaction between cells in the unfrozen fraction and ice and attributed to ice shearing forces or cell deformation. More recently, Ishiguro and Rubinsky (1994, 1998) found that cells caught between the small ice crystals, caused by the presence of antifreeze proteins, were deformed suggesting that ice crystal caused cell deformation as a mechanism of damage during freezing. In multicellular tissues like embryos, extracellular ice crystals within tissues might disrupt cell junctions, and therefore create a plasmalemma lesion through which undercooled cytoplasm might be seeded (Grout and Morris, 1987).

Other factors Farrent (1977) suggested the hypothesis that the damage or survival of living cells during freezing and thawing is primarily linked to bulk water transport across cellular membranes. In addition, certain physical factors such as pressure changes and formation of gas bubbles may also be involved in the destruction of cells (Schneider and Mazur, 1987; Ashwood *et al.*, 1988).

1.2.2.3 Intracellular ice formation

It is an implicit assumption that the formation of ice inside the cell is inevitably lethal. Many studies including Asahina *et al.* (1970), Mazur (1977) and Shabana and McGrath (1988) suggested that intracellular ice formation (IIF) during freezing does correlate with the death of the cells, whilst the specific mechanism of injury and the interactions with ice recrystallization during rewarming are unclear (Mazur, 1977; Fujikawa, 1981). Also there is strong evidence that some cells survive intracellular freezing providing that thawing is extremely rapid, especially over the temperature range of -30 to 0°C (Asahina *et al.*, 1970; Shimada and Asahina, 1975; Griffiths *et al.*, 1979; Acker and McGann, 1999). If thawing over part of this temperature zone is done slowly, cells are killed and this destruction is accompanied by observations of the recrystallisation of ice from the small crystals, produced during rapid cooling, to larger crystals (Shimada and Asahina, 1972). The idea that damage is caused by the large crystals formed by recrystallisation during thawing was put forward by several workers (Rapatz and Luyet, 1963; Moor, 1964; Nei and Asada, 1972; Bank, 1973). Apart from the presence of intracellular ice and the size of each crystal, the total amount of ice is another factor that may be relevant to cell survival (Farrant, 1977; Fujikawa and Miura, 1986). However, recently, *Panagrolaimus davidi*, an Antarctic nematode, has been reported to be the first animal which can survive intracellular freezing as an intact organism (Wharton and Ferns, 1995; Wharton and Block, 1997). The underlying mechanisms of this phenomenon have still to be determined.

There are three possible mechanisms by which IIF can occur: homogeneous nucleation, heterogeneous nucleation, or seeding by the extracellular ice (Franks, 1985). The homogeneous nucleation temperature of a 1 μm droplet of pure water is -39°C, and increases by approximately 2°C for each 10-fold increase in droplet diameter (Wood and Walton, 1970). Solutes depress the homogeneous nucleation temperature by 3.3°C for each

unit increase in solution osmolity. Thus, the expected range of the homogeneous nucleation temperature in cells is -38°C to -44°C (Rall *et al.*, 1983), and for a given cell type it is very narrow. Heterogeneous nucleation relies on the presence of intracellular nucleating agents, evidence for which was provided by Franks *et al.* (1983) who observed IIF at -31 to -38°C in cells cooled in micro-droplet emulsions. The occurrence of IIF at a median temperature of -10 to -20°C in many cell types (Mazur, 1977; Rall *et al.*, 1983) suggests seeding as the predominant mechanism of IIF in many cases.

Understanding the mechanisms of chilling injury and freezing damage helps one to overcome the adverse effects and develop methods that allow low temperature maintenance of living cells, tissues or even organs. Although there was a long history of attempts to cryopreserve living organisms, the first major success occurred fifty years ago when Polge *et al.* (1949) discovered that glycerol provided protection to avian spermatozoa during freezing to -79 °C. Since then significant advances have been made in cryopreservation techniques, which have been widely applied to the storage of many different types of cells and tissues in biological research, clinical medicine and animal breeding. The essence of cryopreservation is to effect cell dehydration and concentration of the cytosol with cryoprotective agents (CPAs) in a controlled manner so that ice crystallisation in the cytosol is precluded or minimised during quenching, commonly in liquid nitrogen (LN_2). There are two approaches to cryopreservation: conventional controlled slow cooling and vitrification. The first important factor which must be considered for both approaches is the selection of the cryoprotectants.

1.2.3 Cryoprotective agents

1.2.3.1 Mechanisms of action of cryoprotectants

There are two major categories of cryoprotective agents: (a) permeating cryoprotectants, e.g. methanol, dimethy sufoxide (DMSO), glycerol and propylene glycol (PG) which are low molecular weight chemicals and can penetrate the cell membrane; and (b) non-permeating cryoprotectants, e.g. hydroxyethyl starch, polyvinyl pyrrolidone and various sugars, which are high molecular weight agents and cannot enter cells. Although the

cryoprotective actions of these different compounds are not entirely understood, cryoprotectants of each group play different roles during cooling and thawing. The permeating cryoprotectants produce a considerable freezing point depression in addition to that due to any electrolytes present within the system, eventually leading to a ternary (cryoprotectants-salt-water) eutectic point at a low temperature (Shepard *et al.*, 1976). In a freezing system, total solute concentration in the unfrozen phase is uniquely determined by temperature. Hence, the inclusion in the system of e.g. DMSO necessarily results in a decrease in concentration of other solutes. As the increasing salt content of the residual liquid is the main cause of so-called 'effect injury' (Mazur, 1965) both the reduction of damaging salt enrichment and its shift to lower temperatures are believed to be beneficial effects. This explanation of cryoprotective properties is also called osmotic buffering. The cryoprotective effect of non-permeating cryoprotectants is mainly based on dehydration of cells prior to cooling, which results in reduced ice crystal formation during freezing. Some high molecular weight (> 50,000) cryoprotectants such as polyvinyl pyrrolidone, polyvinyl alcohol, and hydroxyethyl starch, protect cells during freezing and thawing by altering ice crystal formation to an innocuous size and shape.

1.2.3.2 Toxicity of cryoprotectants

Whilst cryoprotective agents can protect living cells from massive distortions of cellular and environmental geometry, a variety of cryoprotectants can themselves be damaging to cells, especially when used in high concentrations (Fahy, 1986; Arnaud and Pegg, 1988; Pegg and Arnand, 1988; Fahy *et al.*, 1990). Fahy and co-workers (Fahy, 1984; Fahy *et al.*, 1990) have amassed a convincing body of evidence that the basis for the detrimental effects of cryoprotectants is not simply osmotic, but due to direct 'biochemical' injury. Injuries such as inactivation or denaturation of specific enzymes, disruption of transmembrane ionic pumps, or other related perturbations of cellular structure and function, by implication, are most likely due to the direct interaction of the cryoprotectant with proteins and biological membranes. Fahy (1983) found that the addition of cryoprotectant toxicity neutralisers into DMSO before freezing substantially reduced the freezing damage observed after thawing. DMSO appears to interact with the lysine-rich control enzyme for gluconeogenesis, fructose diphosphatase, producing an effective block on glycolysis after DMSO is removed. Cryoprotectant toxicity neutralisers such as urea, formamide, and acetamide might be able to compete the enzyme lysine residues for DMSO binding (Baxter and Lathe, 1971).

1.2.4 Controlled slow freezing

'Controlled slow freezing' evolved from research during the 1950s to the 1970s. During controlled slow cooling procedures, cell dehydration is effected by the freeze-concentration of the suspension medium. The cells are equilibrated in a solution containing a permeating cryoprotective agent, and the suspension is cooled and seeded with ice crystals at a temperature slightly below its freezing point. The specimens were then cooled at an optimum rate to an intermediate subzero temperature prior to quenching in LN_2 so that intracellular ice formation is avoided during ice crystal formation in the suspension medium. The probability of intracellular ice formation is affected by many intrinsic parameters of the specimen (the water and cryoprotectant permeability of the plasma membrane, the surface area to volume ratio of the cell) and the cryopreservation protocol, which includes the type and concentration of the cryoprotectants used, the cooling rate, the seeding temperature, thawing rate, and post-thawing handling.

1.2.4.1 Membrane permeability

Knowledge of the water and cryoprotectant permeability of the membranes of cells and tissues is important not only because the optimal protocols for the addition and removal of cryoprotective solutes or optimal cooling rate for cell cryopreservation can then be modelled (Gao *et al.*, 1995; Mazur, 1963), but also because the fundamental physical mechanisms of solute and water transport across a cell membrane, *e.g.* channels versus lipid bilayer transport can then be characterised (Gutierrez *et al.*, 1995; Yang and Verkman, 1998). A number of formulae are available for determining cell membrane permeability parameters. These include a one-parameter (solute permeability) model (Mazur *et al.*, 1974), a classic two-parameter (water and solute permeability) model (Jacobs, 1932-33), and a three-parameter model developed by Kedem and Katchalsky (1958), which adds a solute solvent interaction term (sigma). The Kedem–Katchalsky (KK) formula has been commonly used by cryobiologists in recent studies. However, the recent discovery and characterisation of water channels (aquaporins) in biological membranes reveals that aquaporins are highly selective for water and do not typically co-transport cryoprotectants. In these circumstances, sigma would be a superfluous parameter. When sigma is not needed, a two-parameter model

utilising only the hydraulic conductivity and solute permeability is sufficient, as it would be simpler to implement and less prone to spurious results (Kleinhans, 1998).

1.2.4.2 Cooling rate

The rate of cooling to final storage temperature can profoundly affect the fate of the biological sample. If cells or tissues are cooled at such a rate that they dehydrate sufficiently to maintain the chemical potential of their intracellular water close to that of water in the partly frozen extracellular solution, such slow freezing is referred to as equilibrium freezing (Mazur, 1990). The quantitative value of equilibrium cooling rates can be computed from the physicochemical differential equations that determine the driving force for efflux and the rate of water loss in response to the driving force (Mazur *et al.*, 1984; Mazur, 1990). At equilibrium cooling rates, cell water can leave to equilibrate with the extracellular ice, and high levels of dehydration are achieved as the solute concentration increases. Increased concentrations of intracellular solutes increase the viscosity of the cytoplasm and depress the equilibrium melting point of the solution, thus decreasing the cytoplasmic supercooling and reducing the likelihood of intracellular nucleation. The dependence of cell dehydration on cooling rate is of practical use, as slow cooling rates can be used to control the concentration and volume of the intracellular solution. However, cell dehydration stops at some characteristic temperature for all cooling rates, because the cell membrane becomes effectively impermeable at sufficiently low temperatures (Kaslsson *et al.*, 1994). Thus, the thermodynamic state of the intracellular solution can be manipulated by dehydration only within a calculable temperature range. Using cooling rates departing from equilibrium freezing, the result is usually intracellular ice formation and cell death, because there is insufficient time for the water to leave the cell. However, if nonequilibrium procedures are used before the temperature has dropped to a level that permits intracellular ice formation, a different result may be obtained. The cell content may then be reduced to the point at which the subsequent rapid nonequilibrium cooling results in either the formation of small innocuous intracellular ice crystals or the conversion of the solution into a glass. High survival may then be achieved depending on warming rates (Mazur, 1990). Although the constant-rate cooling protocol is very commonly used, it has been shown that multistep protocols (piecewise linear) can yield superior results to simple linear methods (McGann and Farrant, 1976). More recent studies on prediction of the effects of candidate

cryopreservation protocols using mathematical models have permitted theoretical optimisation of non-linear protocols (Pitt, 1992; Toner *et al.*, 1993a).

1.2.4.3 Seeding

If ice formation is not induced by seeding, ice will form spontaneously when the medium is cooled to a sufficient degree below its equilibrium melting point. Because ice formation will occur at random, and therefore at unpredictable temperatures, subsequent survival rates will be highly variable between repeated trial with the same freezing protocol. The extremely rapid crystallisation which results when ice forms in a highly supercooled solution can cause damage to cells. It has been shown that the probability of IIF is drastically increased if extracellular ice formation is initiated at high degrees of supercooling (Diller, 1975; Toner *et al.*, 1993a). This phenomenon results from the increased cooling rate experienced by the sample as a consequence of thermal fluctuations caused by the release of latent heat during extracellular ice formation (Mazur,1977), or from the delayed onset of freeze-induced cell dehydration, which results in increased retention of intracellular water, and thus higher probabilities of ice formation in the cell (Diller, 1975).

1.2.4.4 Thawing rate

Cells can be damaged due to crystallisation of ice notably during cooling, but also during warming. Small, innocuous intracellular ice crystals in rapidly cooled cells will grow or agglomerate if warming is too slow, a process referred to as recrystallisation, which can be lethal (Mazur, 1965; Mazur, 1966). Rapid thawing generally improves survival (Farrant, 1980). Shimada and Asahina (1975) have shown that some cells with intracellular ice can survive if thawed very rapidly. Cells cooled at rates faster than the optimal rate which favours intracellular ice formation are more sensitive to a decrease in the rate of thawing than are slowly cooled cells (Mazur *et al.*, 1969; Leibo *et al.*, 1970). Rapid warming results in complete thawing of the suspension before significant embryo dehydration (Rall, 1993). However, there are instances in which slow thawing gives much higher survival than rapid thawing. The study of Zhang *et al.* (1989) on the cryopreservation of common carp (*Cyprinus carpio)* embryos showed that optimum survival of the common carp embryos required warming at a slow rate (8°C/min), since faster rates killed the embryos. Ashwood-

Smith (1980) and Farrant (1980) suggested that injury caused by rapid thawing was possibly due to the presence of a small amount of intracellular ice which seals the cells and results in osmotically induced damaging water movements during the initial part of thawing. Vorotilin *et al.* (1991) suggest that in the course of rapid heating of a frozen biological system, mechanical stresses can affect cells and cause additional damage to them due to the limited thermal conductivity of the system. A two-step warming, a combination of slow warming and rapid warming, was therefore advanced to improve survival (Vorotilin *et al.*, 1991; Pegg *et al.*, 1997).

1.2.4.5 Post-thaw handling

Damaging excessive osmotic swelling will occur when a thawed suspension is diluted with isotonic saline since water moves into the cell to restore osmotic equilibrium. Once equilibrium is restored, the cells gradually shrink as cryoprotectants leave the cytoplasm. The excessive osmotic swelling is prevented by reducing the concentration of cryoprotectant in a series of small steps (Rall, 1993). The appropriate step size and interval between steps is determined by the permeability of the cell membrane to cryoprotectant and water (Schneider and Mazur, 1984). A more commonly used dilution method is the so-called sucrose procedure (Leibo, 1984). The thawed cells or tissues are placed into saline containing a 0.25 to 1 M concentration of sucrose (or other non-permeating solutes) to reduce the transient increase in cell volume by increasing the osmolarity of the suspending solution. As the cryoprotectant leaves the cytoplasm, the cell progressively shrinks due to the hypertonic extracellular solution. Once the cryoprotectant leaves the cells, the suspending solution is replaced with isotonic saline (Rall, 1993). Robertson *et al.* (1988) reported that Red Drum morulae exposed to 0.1 M sucrose showed significantly improved hatch and survival rates.

All the above interacting variables must be simultaneously optimised according to the parameters characteristic of the specimen before an effective cryopreservation protocol can be designed. It is a time-consuming task if the cryopreservation of a large number of different specimens, for instance organs or differentiated embryos, is under consideration.

1.2.5 Vitrification

Vitrification refers to the transformation of a liquid into a glass. It occurs when the viscosity of the solution reaches a sufficient value (arbitrarily set at $10^{14.6}$ Poises) and crystallisation is inhibited (Pegg and Diaper, 1990). Vitrification of a biological specimen means that the cells as well as the extracellular solution are supercooled to the glass transition temperature and solidified into the amorphous or glassy state. During vitrification molecular motions are significantly arrested, marking the effective end of biological time but without any of the changes brought about by freezing. Although Luyet (1937) was one of the first to consider vitrification for purposes of cryopreservation, and studied this approach intensively over a number of years with emphasis on the conditions under which cryobiologically relevant aqueous solutions would or would not vitrify (Luyet, 1966), the method received little attention until the report of Rall and Fahy (1985) on the successful cryopreservation of mouse embryos by vitrification.

1.2.5.1 Vitrification procedures

In vitrification procedures, cell dehydration is effected by direct exposure to concentrated vitrification solutions prior to cooling in LN_2. Under ideal conditions, the cells or tissues can be cooled at extremely rapid rates without undergoing intracellular ice formation. Operationally, procedures for vitrification and recovery usually consist of five steps (Steponkus *et al.*, 1992): a) equilibration of the cells or tissues in a permeating cryoprotectant solution; b) dehydration of the cells or tissues in a concentrated solution that will vitrify (vitrification solution); c) plunging the cells or tissues in LN_2; d) warming the cells or tissues; e) dilution of the vitrification solution and removal of cryoprotectants from cytosol.

Equilibration To effect vitrification of the intracellular solution during quenching in LN_2, it is necessary to increase the solute concentration of the cell. This can be facilitated by introducing a moderate concentration (1 - 2 M) of permeating cryoprotectants into the cell. This is usually done at room temperature to minimise the time required for equilibration.

Dehydation Before plunging into LN_2, the cytosol must be concentrated to the level required for vitrification by dehydrating the cells in a vitrification solution, which is an

extremely concentrated (normally > 8 M) solution of cryoprotectants. This step is usually carried at 0 - 4°C to minimise the injurious consequences of the exposure to the vitrification solution because this is potentially the most injurious step in the entire vitrification procedures.

Cooling/Warming Typically the cells are cooled by quenching in LN_2 and warming is effected by plunging the cells into warm water or other fluids. However, LN_2 is not the most effective cryogenic fluid because vaporisation of the LN_2 and the formation of gas bubbles around the sample impedes heat transfer. Nitrogen slush or liquid propane supercooled with LN_2 are therefore sometimes applied to effect more rapid cooling (Steponkus *et al.*, 1990; Mazur *et al.*, 1993). Higher warming rates are also required to prevent devitrification, which would result in the recrystallisation of the vitrified solution (Mazur *et al.*, 1993).

Dilution Dilution of the vitrification solution should be done as soon as possible following warming because of the extremely high concentrations of cryoprotectants used for vitrification. Like the post-thaw handing in slow cooling procedures, this step is usually accomplished by dilution in a hypertonic medium (e.g. 0.5 - 1 M sucrose) or a stepwise dilution in order to minimise osmotic expansion.

The goal of vitrification procedures is to preclude ice formation in both intracellular and extracellular solutions and still maintain high levels of viability. Whether or not ice formation occurs primarily depends on the composition of the vitrification solution and the cooling and warming rate used. Sufficiently concentrated solutions of most cryoprotectants will vitrify, even at slow cooling and warming rates. Unfortunately, at the concentration required for vitrification of most cryoprotectants, the toxicity of the solutions is manifested during the dehydration step because of the extremely high osmotic potentials of the solutions (Steponkus *et al.*, 1992). Formulation of a species specific, less toxic and effective vitrification solution is therefore essential for successful vitrification.

1.2.5.2 Vitrification solutions

Two requirements should be satisfied in formulating a successful vitrification solution (Fahy *et al.*, 1984; Rall, 1987): the first is related to the physicochemical (glass-forming tendency) properties of the vitrification solution - it must be sufficiently concentrated to

avoid crystallisation during cooling and vitrify into a glassy solid; the second is to match the choice of cryoprotectants with the intrinsic permeability and toxicity properties of the cells in question. Ideally, at least one of the cryoprotectants in the vitrification solution should permeate the cytoplasm, but the overall composition must not produce excessive osmotic stress or chemical toxicity (Rall, 1987).

Stability of amorphous state and glass-forming tendency The amorphous state may be defined by the critical warming rate at which the difference between devitrification and melting temperature vanishes. Under such circumstances, ice formation and the concomitant damaging salt enrichment are completely avoided during both cooling and subsequent warming and it is evident that the more stable the amorphous state is, i.e., the lower the critical warming rate, the better the cryoprotective action. Fahy *et al.* studied the 'concentration needed to vitrify', which they defined as the minimum concentration of a cryoprotectant required to achieve vitrification (Fahy and Hirsh, 1982; Fahy *et al.*, 1984). Meanwhile, Boutron and Kaufmann (1979, 1987) investigated non-equilibrium freezing, the stability of the amorphous state, and the glass-forming tendency of polyalcohols. PG was found to have better glass-forming properties than many other polyalcohols: 45 % (w/w) PG in water vitrified at rates as low as 10°C/min whereas the same concentration of glycerol needed to be cooled at 300°C/min to vitrify. These results were in agreement with Sutton's study (1990) on time-temperature transformation and continuous cooling curves for PG using isothermal emulsion calorimetry. Later, butane-2,3-diol was reported to be able to vitrify at a lower concentration of 35% (w/w) when cooled at only 20°C/min compared with the 200 - 300°C/min required to vitrify the same concentration of PG (Boutron, 1990). The studies by Boutron *et al.* on PG and butane-2,3-diol (Boutron, 1987, 1990, 1992) suggested the possible use of these diols as vitrification agents, if one avoids use of the meso-form of butane-2,3-diol, which crystallises as a stable hydrate. The addition of sugars, such as sucrose, raffinose and trehalose, polyvinylpyrrolidone or polysaccharides has been shown to improve the glass-forming tendency and reduce the critical cooling rates for aqueous penetrating cryoprotectants (Sutton, 1991, 1992; Kuleshova *et al.*, 1999). It has also been reported that modifying conventional polyol cryoprotectants by substituting methoxyl ($-OCH_3$) groups for hydroxyl (–OH) groups on cryoprotectant molecules reduces the viscosity, increases the permeability and the glass-forming tendency of cryoprotectant solutions (Wowk *et al.*, 1999).

Toxicity of vitrification solutions Cryoprotectant toxicity has been considered as "a subject which arguably could be considered the central problem of cryobiology" (Fahy, 1987). Fahy has considered whether the toxicity of the vitrification solutions is a result of either 'osmotic' or 'biological' effects, with the assumption that 'osmotic' effects are the result of volumetric changes that occur during osmotic excursions and 'biochemical' effects are responsible for all other manifestations of injury (Fahy *et al.*, 1984; Fahy, 1987; Fahy *et al.*, 1987). Fahy concluded that the toxic effects are biological and that injury is a consequence of concentration of cryoprotectants *per se*. However, an osmotic stress, especially of the magnitude incurred in the concentrated solutions typically used for vitrification, involves more than just volumetric excursions. Severe dehydration resulting from large osmotic potentials results in structural transitions in a diverse array of biological molecules including lipids, proteins, and nucleic acids (Parsegian *et al.*, 1986). These transitions occur because the large osmotic pressure is sufficient to overcome the strongly repulsive hydration forces that are associated with hydrophilic surfaces of macromolecules. Therefore, the toxicity of vitrification was also considered to be a consequence of severe dehydration resulting from the large osmotic stresses imposed during the dehydration step (Steponkus *et al.*, 1992).

Approaches to reducing the toxicity of vitrification solutions There are several approaches to the development of less toxic vitrification solutions. One way is to formulate a solution that is a mixture of several different components, with the assumption that the solution will be less toxic because the concentration of any one component is decreased (Fahy *et al.*, 1984, Scheffen *et al.*, 1986). The inclusion of sugars in vitrification solutions has been found to reduce the toxicity of cryoprotectants (Boutron and Peyridieu, 1994; Kuleshova *et al.*, 1999). Another approach is to add components that act as toxicity neutralisers or protectors against injury resulting from the other components (Fahy, *et al.*, 1987). For example, acetamide, which was used in the original vitrification solutions of Fahy, is considered to be a toxicity neutraliser that mitigates the toxic effects of DMSO (section 1.2.3.2). A third approach is to identify solutes that will form a glass at the lowest concentration. Considering the damaging osmotic stresses imposed on cells during exposure to the vitrification solution, an alternative strategy for formulating less toxic vitrification solutions is to optimise the osmolality of the vitrification solution, with the optimum being

that which results in the minimum amount of cell dehydration required for the cytosol to form a stable glass during the subsequent cooling/warming steps (Steponkus *et al.*, 1992).

1.2.5.3 Advantages and disadvantages of vitrification

Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because potential injury associated with ice formation in the suspension is obviated. There are several advantages of this approach: (1) offering greater potential for developing cryopreservation procedures for complex tissues and organs, (2) circumventing problems of chilling sensitivity of some specimens, (3) requiring less specialised or expensive equipment. So far vitrification has been successfully applied to the preservation of tissues (Armitage, 1986; Jutte *et al.*, 1986), blood cells (Boutron, 1992), plant somatic embryos of *Asparagus officinalis* (Uragami *et al.*, 1989), *Drosophila melanogaster* embryos (Steponkus *et al.*, 1990; Mazur *et al.*, 1992a) oocytes and embryos of mouse (Hsu *et al.*, 1986; Scheffen *et al.*, 1986; Bielanski, 1987; Friedler *et al.*, 1987; Kono and Tsunoda, 1987; Rall, 1987; Rall *et al.*, 1987; Nakagata and Douglas, 1989; Shaw and Trounson, 1989; Kasai *et al.*, 1990; Valdez *et al.*, 1990; Shaw *et al.*, 1991), hamster (Critser *et al.*, 1986), rat (Kono *et al.*, 1988), rabbit (Smorag *et al.*, 1989; Kobayashi *et al.*, 1990), goat (Yuswiati and Holtz, 1990), sheep (Gajda *et al.*, 1989; Schiewe *et al.* 1990 ; Szell *et al.*, 1990), cow (Massip *et al.*, 1986; Van *et al.*, 1989; Vajta *et al.*,1998), pig (Rubinsky *et al.*, 1991; Nagashima *et al.*, 1996), and human (Trounson, 1986).

However, several uncertainties remain in the use of vitrification including cellular tolerance of highly concentrated cryoprotectant solutions, cooling and warming of cells at relatively high rates, thermal shock, and devitrification (ice formation during warming), fracturing and crystallisation during cooling of large biological systems (Fahy *et al.*, 1984; Armitage and Rich, 1990; Fahy *et al.*, 1990a; Sutton, 1991). Since the concentrations of solutes involved in achieving a vitrifiable solution are substantial, typically in excess of 30 % (w/v), the toxicity of the solutes is also always a serious consideration (MacFarlane and Forsyth, 1990). Moreover, some specimens, like most insect embryos and teleost embryos, may have a low plasma membrane permeability to cryoprotectants and water, and this will result in insufficient cell dehydration and permeation by cryoprotectants into the cells or tissues. Vitrification will not be achieved if the low permeability of the membrane can not be surmounted in advance.

1.3 Attempts to cryopreserve fish embryos

In the last 20 years, attempts to cryopreserve fish eggs and embryos have been conducted on 10 species including herring (*Clupea harengus*) (Whittingham and Rosenthal, 1978; Ben-Amotz and Rosenthal, 1981), rainbow trout (*Salmo mykiss*) (Haga, 1982), brown trout (*Salmo trutta*) (Erdahl and Graham, 1980), brook trout (*Salvelinus fontinalis*) (Zell, 1978), coho salmon (*Onorhynchus kisutch*) (Stoss and Donaldson, 1983), Atlantic salmon (*Salmo salar*) (Zell, 1978; Harvey and Ashwood-Smith, 1982), common carp (*Cyprinus carpio*) (Zhang *et al.*, 1989), medaka (*Oryzias latipes*) (Onizuka *et al.*, 1984), African catfish (*Clarias gariepinus*) (Magyary *et al.*, 1996) and zebrafish (*Danio rerio*) (Harvey, 1983; Zhang *et al.*, 1993; Zhang and Rawson, 1996a). Controlled slow cooling was employed in most of these studies. Although eggs or embryos have been shown to survive for a short time after cooling to subzero temperatures, successful cryopreservation of fish eggs and embryos remains elusive.

1.3.1 Attempts to cryopreserve fish embryos using controlled slow cooling

Controlled slow cooling of fish embryos involved the selection and the optimisation of the following factors: (a) embryo developmental stage; (b) cryoprotectants - type, concentration, treatment time and methods; (c) cooling rates; (d) thawing rates and methods; (e) removal of cryoprotectants.

Embryo developmental stage Because of the stage-dependent chilling sensitivity (Haga, 1982; Cloud *et al.*, 1988; Liu *et al.*, 1993; Zhang and Rawson, 1995; Dinnyes *et al.*, 1998) and the stage-associated permeability of fish embryos (Zhang and Rawson, 1998), the selection of the right embryo stage appears to be the first important step in the cryopreservation of fish embryos. Intermediate embryo development stages between post-gastrula and heart beat, which are less sensitive to chilling than earlier stage embryos, have been reported to have higher survival rates after cooling to subzero temperatures. For example, embryos at tail-bud stage in common carp (Zhang *et al.* 1989), post-eyed stages in

rainbow trout (Haga 1982) and 6-somite to heart beat stages in zebrafish (Zhang *et al.*, 1993) were showed to be the optimum stages for survival following cooling to -30°C.

Cryoprotectants - type, concentration, treatment time and methods The selection of cryoprotectants and protocols is considered to be species related, and requires an understanding of their toxicity and ability to permeate the embryos (Rall, 1993). The most commonly used cryoprotectant in fish egg and embryo preservation is DMSO, which has been applied to the cryopreservation studies of herring (Whittingham and Rosenthal, 1978; Ben-Amotz and Rosenthal, 1981), rainbow trout (Haga, 1982; Harvey and Ashwood-Smith, 1982), chum salmon (Harvey and Ashwood-Smith, 1982) coho salmon (Stoss and Donaldson, 1983), medaka (Onizuka *et al.*, 1984), and common carp (Zhang *et al.*, 1989) eggs or embryos. However, for zebrafish embryo cryopreservation, DMSO has been found to be ineffective (Harvey, 1983), and methanol was shown to be more effective than DMSO (Zhang *et al.*, 1993). This is mainly attributable to the relatively higher penetration and lower toxicity of methanol to zebrafish embryos (Zhang *et al.*, 1993; Zhang, 1994, Hagedorn *et al*, 1997a). The concentrations of cryoprotectants used for slow cooling of fish embryos are in the range of 1 - 3 M. Embryos are normally treated with cryoprotectants at room temperature or 0°C for 0.5 to 1 h in either a one step or multi-step addition.

Cooling rates Slow cooling rates normally result in higher survival for fish embryos when compared to fast cooling rates due to the low permeability of these embryos to water and cryoprotectants, resulting from the large size of the fish eggs and the presence of permeability barriers (see Section 1.4.1). The cooling rates employed in the controlled slow cooling procedures for fish embryos have been reported to be in the range of 0.01-0.75°C/min (Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983; Onizuka *et al.*, 1984; Zhang *et al.*, 1993). The optimum cooling rate for the cryopreservation of zebrafish embryos (heart-beat stage) has been found to be 0.3°C/min by comparing a range of cooling rates (0.1 - 0.75°C/min).

Thawing rates and methods Optimum thawing rates reported for fish embryos vary, although a slow rate (8°C/min) was optimal for common carp embryos (Zhang *et al.*, 1989), an intermediate rate (43°C/min) was preferred for zebrafish blastoderm (Harvey, 1983) and a fast rate (300°C/min) resulted in best survival for zebrafish embryos (Zhang *et al.*, 1993).

Removal of cryoprotectant The use of sucrose in the diluting medium to allow controlled removal of cryoprotectants to avoid osmotic damage, is reported to improve the survival of common carp (Zhang *et al.*, 1989) embryos after cooling whilst no difference was observed for zebrafish embryos (Zhang *et al.*, 1993).

Fish eggs or embryos from all species have shown a certain tolerance to temperatures below 0°C, but limited success in terms of hatching has been reported on cooling to -25°C or lower even with the optimum slow cooling protocols. Fish embryos do not normally survive cooling to -35°C. Although several common carp embryos were reported to survive cooling to -196°C and hatch (Zhang *et al.*,1989), these results have not been duplicated elsewhere. The loss of viability has been considered to be mainly due to the intracellular ice formation (Harvey and Ashwood-Smith 1982; Chen *et al.*, 1988; Zhang *et al.*, 1993), but it may also be attributable to the chilling injury of the embryos, which was not to be distinguished from the intracellular freezing injury in these studies. The studies on kinetics of subzero chilling injury in *Drosophila* embryos (Mazur *et al.*,1992) and chilling sensitivity of zebrafish embryos (Zhang and Rawson, 1995) have demonstrated that chilling injury plays an important role in the reduction of embryo survival during exposure to subzero temperatures.

1.3.2 Attempts to cryopreserve fish embryos using vitrification

There have been only two reports on the feasibility of cryopreservation of fish embryos using vitrification. Wang *et al.* (1987) examined the toxicity of the first vitrification solution (VS1), which was origirally developed by Rall and Fahy (1985) for mouse embryo cryopreservation, and PG on fish embryos at 5°C and 20°C. They found that concentrations above 50 % of VS1 and 35 % (v/v) of PG had detrimental effects to fish embryos due as much to osmotic stress as to toxicity. Cryomicroscopy observations showed that fish embryos exposed to 100 % VS1 and 45 % (v/v) PG for 20 min did not crystallise either intracellularly or extracellularly during cooling to -130°C when the cooling rate was higher than 20°C/min. However, during rewarming, intracellular blacking appeared at -80 to -60°C, which is usually recognised as devitrification and may injure fish embryos. By testing the vitrification characteristics and toxicity of a number of cryoprotectants and vitrification

solutions, Zhang and Rawson (1996a) found that vitrification solution BPP (2 M butane-2,3-diol + 3 M PG + 6% polyethylene glycol 400) was least toxic to zebrafish embryos and remained transparent during rapid cooling in LN_2 (40 µl BBP loaded in 0.25-ml straws and plunged into LN_2) and rapid warming.

Vitrification of intact zebrafish embryos in straws (Zhang and Rawson, 1996a) showed that after two-step treatment procedures (2 M PG for 30 min followed by BPP for 5 min at room temperature) about 32% of 6-somite and 25% of heart-beat stage embryos remained morphologically intact immediately after thawing, but collapse of vitelline membrane and rupture of the yolk of these embryos were observed within 15 min after dilution of cryoprotectants. The inevitable intraembryonic ice formation during cooling resulting from the low degree of cryoprotectant permeability of the embryos was considered to be directly responsible for the lethal injury during vitrification procedures (Zhang and Rawson, 1996a).

1.4 Factors limiting the successful cryopreservation of fish embryos

Whilst successful cryopreservation of embryos has been reported for more than one dozen species of mammals and a few non-mammalian species (Rall, 1993), cryopreservation of fish eggs and embryos has posed several problems associated with the injuries induced during the cooling and/or the thawing processes and three major characteristics in particular have been identified as possibly being responsible.

1.4.1 Low permeability to water and cryoprotectants

Removal of most of the osmotically active water within embryos to achieve sufficient permeation of cryoprotectants into the embryos is crucial to prevent intracellular ice formation and aid successful cryopreservation. Unfortunately, fish embryos have been found to have low permeability to both water and most cryoprotectants (Loeffler and Lovstrup, 1970; Zhang and Rawson, 1996, 1998; Hagedorn *et al.*, 1997a, 1997b). One reason for this low permeability is the large overall size of fish embryos. The eggs of most fish species are greater than 1 mm in diameter (previously successfully cryopreserved eggs are all considerably smaller: mammalian eggs, 70 - 150 µm; invertebrate eggs, < 300 µm in

diameter). This large size results in a much lower surface area to volume ratio. One consequence of the low ratio is a reduction in the rate at which water and cryoprotectants can move into and out of the embryo during the steps of cryopreservation (Mazur, 1984). Permeability barriers such as the yolk syncytial layer (YSL) in zebrafish embryos has also been reported to account for the observed low cryoprotectant permeability of fish embryos (Hagedorn *et al.*, 1996).

1.4.2 High chilling sensitivity

Stage-dependent chilling sensitivity has been reported for many species of fish embryos, including brown trout (Meddock, 1974); rainbow trout (Haga, 1982); carp (Jaoul and Roubard, 1982; Roubaus *et al.*, 1985; Dinnyes *et al.*, 1998) fathead minnows (Cloud *et al.*, 1988); goldfish (Liu *et al.*, 1993) and zebrafish (Zhang and Rawson, 1995). Most of these studies reveal that developmental stages beyond 50%-epiboly are less sensitive to chilling, but the chilling sensitivity accelerates rapidly at subzero temperatures (Zhang and Rawson, 1995). The reason for the extent of stage-dependent chilling sensitivity might be related to the changes in cell and tissue types, number of cells, effectiveness of repair mechanisms and enzymatic reactions. Roubaud *et al.* (1985) suggested that the progressive acquisition of tolerance to cold shock of carp embryos may be related to the slowing down and desychronisation of cell division cycles at later stages. Although the presence of cryoprotectants somewhat mitigates the chilling injury in fish embryos (Zhang and Rawson, 1995; Dinnyes *et al.*, 1998), the reduction in subzero chilling injury is limited (Zhang and Rawson, 1995). Studies with *Drosophila* embryos showed that chilling injury in permeabilised embryos containing ethylene glycol was roughly comparable to that in intact embryos which contained no cryoprotectants (Leibo *et al.*, 1988; Mazur *et al.*, 1992). The exact physiological process responsible for the chilling injury in fish embryos is not well understood. Mazur *et al.* (1992) hypothesised that the high chilling sensitivity of these embryos may result from the loss of synchrony of coupled reactions involved in embryological development. Studies involving mammalian embryos suggest that a high sensitivity to chilling injury is associated with large amounts of intraembryonic lipids (Polge *et al.*, 1974; Mohr and Trounson, 1981; Toner *et al.*, 1986; Nagashima *et al.*, 1994) which are also commonly present in fish embryo yolk and cell compartments. It needs to be determined whether or not the high chilling sensitivity of fish embryos is related to the

intraembryonic lipids. The high chilling sensitivity precludes the application of the conventional controlled slow cooling procedures to the cryopreservation of fish embryos, at least for some species.

1.4.3 Two compartment nature of the fish embryo with a high yolk content

Dechorionated fish embryos have two compartments: the blastoderm and yolk. The high yolk content provides all nutrients for embryonic development. During the cryopreservation, the yolk probably acts as an independent compartment and responds osmotically in a manner analogous to the cellular cytoplasm. The development of a single effective protocol for cryoprotectant permeation and osmotic dehydration of both the yolk and cells of the embryos may be difficult due to the distinctive volume difference of the two compartments (Rall, 1993).

1.5 Zebrafish (*Danio rerio*) embryo model system for fish embryo cryopreservation

Zebrafish (D*anio rerio*) embryos were used as a model system for fish embryo cryopreservation as they exhibit the following ideal model features (Rall, 1993): (1) small adult size; (2) short generation interval; (3) both fish and embryos can be maintained *in vitro* throughout the entire life cycle; (4) eggs, embryos and spermatozoa can be available daily (i.e. non-seasonal breeder); and (5) appropriate reproductive biotechniques are available (e.g., oocyte, semen and embryos collection, embryo micro-manipulation and survival surgery). The zebrafish model has also become one of the most important vertebrate models for the study of development and genetics (Kaha, 1994). Transgenic and mutagenic studies of zebrafish are expected to play an important role in understanding human health and disease. The human genome is being mapped, but functionality of many of the genes is not known. On the other hand, the function of many of the genes in the zebrafish genome is known because of the mutational screening underway in a number of laboratory throughout the world. When the zebrafish genome is fully mapped, it will have great value, because the organisation of the fish and human genomes has been relatively highly conserved throughout evolution (Postlethwait *et al*, 1998). Such studies require large

numbers of mutant zebrafish to be maintained. The ability to cryobiologically preserve zebrafish embryos would therefore be a great service to this field.

1.5.1 Zebrafish reproduction

Zebrafish *Danio rerio* are tropical Cypriniform, representative of the family *Cyprinidae*. They are freshwater fish that were originally found in slow streams and rice paddies and in the Ganges River in East India and Burma (Axelrod & Schultz, 1955). The fish rarely exceeds 4.5 cm in length and has a cylindrical body with 7-9 dark blue horizontal stripes on silver, which run into caudal and anal fins, and an olive green back. Males are slimmer than the females and possess a golden sheen, whilst females are more silvery and their abdomen is distended particularly prior to spawning. The fish are capable of withstanding wide ranges of temperature (15.5 - 43.3°C) and pH (6.6 - 9.2) (Axelrod and Schult, 1955).

The zebrafish is an oviparous species. The average weights of a three month old female and male were 1100 mg and 600 mg respectively (Piron, 1978). Hisaoka and Firlit (1962) have reported on the oviposition and ovarian cycle in adult zebrafish. Eggs are laid in large numbers within a period of 5 - 45 days following the previous oviposition, the optimal time being from 5 - 10 days with eggs not usually laid on the first 4 days after the previous oviposition. Mortality and abnormality due to over ripeness occurs when the eggs are laid after a rest period of 15 days. The maximum number of eggs obtainable from a single female may be as high as 1500 - 1800, whilst the number laid generally varies between 150 and 400 eggs per spawning. The influence of breeding time interval on egg number, mortality and hatching was also investigated by Niimi and LaHam (1974). According to their report a breeding interval of 10 days ensured low mortality and uniform hatching at 26 ± 1°C. Mortality increased from 5 to 100 % as the time interval between breeding period increased from 10 - 45 days (Laale, 1977). Piron (1978) also found that intensive inbreeding of this fish gave rise to a high incidence of spontaneous skeletal deformities.

The species has been studied extensively since the nineteen thirties in fisheries research because it is easily obtainable, inexpensive, readily maintained and cared for (Rugh, 1948), undergoes a photoperiodic response which allows the time of spawning to be controlled (Legault, 1958) and, under appropriate laboratory conditions, will provide large numbers of non-adherent and transparent eggs throughout the year (Hisaoka, 1958). Since George

Streisinger (1981) first recognised that the zebrafish is a good model for studying vertebrate development and genetics, the zebrafish has become very popular world-wide as a means of understanding how not only fish, but all vertebrates including people, develop from the moment that sperm fertilises an egg. The eggs are transparent and develop outside of the mother's body, allowing the development from fertilisation to a newly formed fish to be observed under a microscope.

1.5.2 Structure of zebrafish embryos

The intact zebrafish embryo consists of the chorion, perivitelline space, and the developing embryo. The developing embryo has two compartments: blastoderm and yolk, which are surrounded by the plasma (vitelline) membrane. Underlying the cellular blastoderm and covering the yolk is the yolk syncytial layer, which develops at approximately the 1,000-cell stage of development (Fig. 1.2).

Embryo membranes In zebrafish embryos, two distinct membranes are recognised; namely, the outer chorionic membrane (zona radiata) and the inner plasma (vitelline) membrane (Hisaoka, 1958; Schmehl and Grauham, 1987). The studies of fine structure of

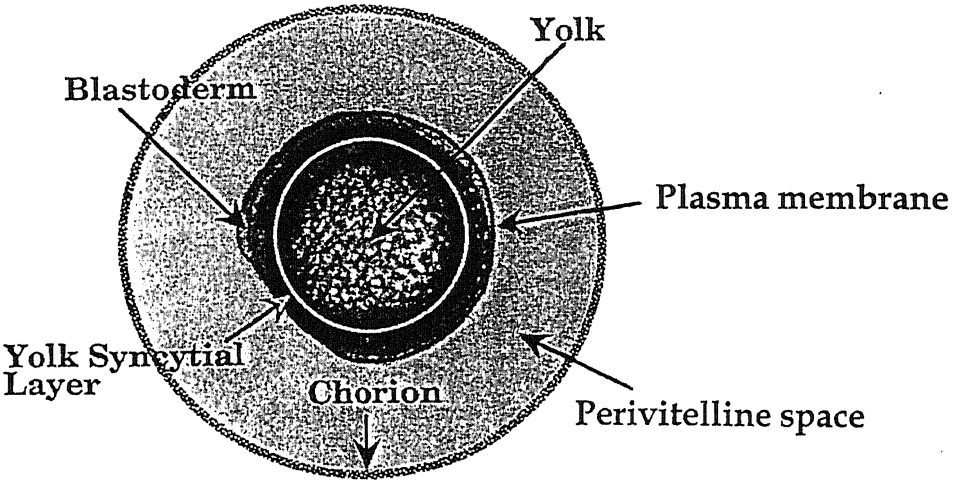


Fig. 1.2 Image of a typical intact zebrafish embryo at 6-somite stage, showing the structure of zebrafish embryos. The yolk syncytial layer would not be visible in this image, but its position is included for clarity (modified from Hagedorn *et al.*, 1996).

the chorion (Hart and Donovan, 1983) showed it to be a thin envelope, approximately 1.5 - 2.0 μm in thickness, constructed of three distinct zones: an outer, electron-dense zone containing pore canal plugs (zona radiata externa) rich in polysaccharides (Tesoriero, 1977), a middle fibrillar zone (superfical zona radiata interna), and an inner zone (deep zona radiata interna). It is generally believed that the zona radiata interna is rich in proteins (Yamagami, 1981). Such a structure may play a role in diffusive exchange of gases as well as providing physical protection (Stehr and Hawkes, 1979; Grierson and Neville, 1981). It also plays a role as a flexible filter for transport of some materials (Toshimore and Yasuzumi, 1976) and protects against micro-organisms (Schoots *et al.*, 1982). Studies on the chorion permeability (Hisaoka 1958, Zhang and Rawson, 1996a) of the zebrafish eggs demonstrated it to be a porous membrane freely permeable to water, electrolytes and a range of cryoprotectants. The plasma membrane of the teleost embryos is a conspicuous and prominent region and the organisation of these membrane includes an actin-containing cytoskeleton which may function to maintain the shape of the egg and its surface specialisation (Hart, 1990). The plasma membrane permits gas exchange but it, and/or the underlying syncytial layer, appear to be relatively impervious to most solutes (Heming and Buddington, 1988). Studies of the plasma membrane of zebrafish embryos have showed that the permeability of this membrane to water and most cryoprotectants is low (Zhang and Rawson, 1996, 1998; Hagedorn *et al.*, 1997a, 1997b). The plasma membrane of gastrula stage embryos has recently been found to have three morphologically distinct regions, being prominently ridged and folded at the surface of the blastoderm, smooth over the syncytial layer at the vegetal pole, and with an intermediate region between the animal and vegetal pole where folding develops in advance of the expanding blastodermal disc of cells (Rawson, *et al.*, 2000). These structural differences may provided some explanation of the different osmotic properties reported for blastoderm and yolk compartments (Hagedorn *et al.*, 1996). The ridge-like folds covering the blastoderm surface provide a larger surface/volume ratio which may confer greater membrane permeability to water and solutes.

Perivitelline space Water activation of a teleost egg results in a small amount of fluid and protein filling the space between the chorion and vitelline membrane. The perivitelline fluid is produced when water flows across the chorion in response to an osmotic pressure gradient between the external water and the colloids (Kalman, 1959). The formation of perivitelline fluid is brought about by the release of colloid and protein into the perivitelline space, which imbibes water from the external medium, a process known as 'water harding'

(Pott and Eddy, 1973). Eddy (1974) found the perivitelline fluid of the Atlantic salmon (Salmo salar) egg to consist of 58% water, 25% protein, 12% lipid, and 1.7% carbohydrate. The perivitelline fluid and its associated multilamellar envelopes, with their varied specialised patterns and filamentous elaboration, provide a number of protective, nutritive, flotative, polyspermy preventive and regulative functions (Laale, 1980).

Yolk The structural components of fish yolk include yolk platelets and oil globules. Yolk platelets are composed of mucopolysaccharides, lipovitelline and phosvitin or analogous lipoproteins and phosphoproteins (Heming and Buddington, 1988). Ulrich (1969) has described two main phases in the evolution of the oocyte ultrastructure of zebrafish. The first phase - previtellogenesis, is characterised primarily by growth, and the termination of this phase by elaboration of cortical Golgi complexes in which mucopolysaccharide yolk vesicles are synthesised. The second phase, vitellogenesis, is characterised by yolk accumulation and is the phase during which protein yolk globules are formed by a fusion of pincytotic vesicles. The yolk, besides providing solute nutrient to the cells of the rapidly developing blastoderm, contributes ribosomes, and possibly membranous material as well, to the embryonic cells for their subsequent differentiation (Thomas, 1966; Thomas, 1968; Dasgupta and Singh, 1981; Kimmel and Law, 1986).

Yolk syncytial layer (YSL) The YSL is a multinucleate layer (ca. 10-μm thick) of nonyolky cytoplasm underlying the cellular blastoderm and covering the yolk. It forms at the time of the 10th (sometimes 9th) cell division by a collapse of a set of blastomeres, termed marginal blastomeres, into the yolk cell (Kimmel and Law, 1985). During development, the YSL replaces the thin (ca. 2-μm thick), non-nucleated yolk cytoplasmic layer (Betchaku and Trinkhaus, 1978). At first the YSL has the form of a narrow ring around the blastodisc edge, but soon (within two division cycles) it spreads underneath the blastodisc, forming a complete "internal" syncytium (the I-YSL), that persists through embryogenesis. In this position, between the embryonic cells and their yolk stores, the I-YSL might be presumed to be playing a nutritive role. Another portion of it, the E-YSL, is transiently "external" to the blastodisc edge during epiloly. The E-YSL appears to be a major motor for epiboly (Trinkaus, 1984). The YSL has recently been reported to be a permeability barrier to cryoprotectants in zebrafish embryos (Hagedorn *et al.*, 1996).

Blastoderm This is the developing part of the embryo. The volume and shape changes rapidly throughout development. The first cleavage occurs about 40 min after fertilisation and subsequently the blastomeres divide at approximately 15-min intervals. The cytoplasmic divisions are meroblastic and discoidal. At the 128-cell stage, the blastodics begins to look ball-like, and the blastoderm is then converted from the blastodisc by epiboly during the blastula period. After about 10 h development at 28.5°C, a variety of morphogenetic movements occur in the blastoderm: the somites develop, the rudiments of the primary organs become visible, the tail bud becomes prominent and the embryo elongates (Kimmel *et al.* 1995). Subsequent development is detailed in Table 1.1.

1.5.3 Developmental stages of the zebrafish embryos

Zebrafish development varies as a function of incubation temperature. The embryos appear to develop normally if they are kept between 23 and 34°C (Schirone and Gross, 1968). Their development is relatively fast; at 26°C hatching takes place after approximately 4 days and the time from fertilisation to yolk absorption by the larvae is approximately 11 days (Hisaoka and Battle, 1958). Recently, Kimmel *et al.* (1995) described the series of stages in development of dechorionated zebrafish embryo based on the 'standard developmental time', often designated by the letter 'h', defined as normalised hours after fertilisation at 28.5°C (Table 1.1). Fig. 1.3 showed six selected developmental stages of zebrafish embryos which were mainly used in the present study. Within the range of 25 and 33°C, the relationship between the development and temperature is approximately linear, and the following relation allows one to determine approximately when embryos, developing at any temperature within this range, will reach a desired stage of interest (Kimmel, 1994):

$$H = h / (0.055T - 0.57)$$

where H = hours of development at temperature T, and h = hours of development to reach the stage at 28.5°C.

Table 1.1 Stages of zebrafish embryo development at 28.5 °C (from Kimmel *et al.*, 1995)

Stage	h[a]	HB[b]	Description
Zygote period			
1-cell	0	1,2	Cytoplasm streams towards animal pole to form the blastodisc
Cleavage period			
2-cell	3/4	3	Partial cleavage
4-cell	1	4	2 × 2 array of blastomeres
8-cell	1 1/4	5	2 × 4 array of blastomeres
16-cell	1 1/2	6	4 × 4 array of blastomeres
32-cell	1 3/4	7	2 regular tiers[c] of blastomeres, sometimes present in a 4 × 8 array
64-cell	2	8	3 regular tiers of blastomeres
Blastula period			
128-cell	2 1/4	9	5 blastomeres tiers; cleavage planes irregular
256-cell	2 1/2		7 tiers of blastomeres
512-cell	2 3/4		9 tiers of blastomeres; Nomarski optics (NO): yolk syncytial layer (YSL) forms
1 k-cell	3	10	11 tiers of blastomeres; NO: YSL nuclei in a single row; slight cell cycle asynchrony
High	3 1/3		>11 tiers of blastomeres; substantial division asynchrony and beginning of blastoderm flattening; NO: YSL nuclei in two rows; blastoderm beginning to flatten
Oblong	3 2/3	11	Flattening produces an eliptical shape; NO: multiple rows of YSL nuclei
Sphere	4	12	Spherical shape; flat border between blastodisc and yolk
Dome	4 1/3	13	Blastula remains spherical; yolk cell bulging (doming) towards animal pole as epiboly begins
30%-epiboly	4 2/3	14	Blastoderm shaped as an inverted cup of uniform thickness; margin reaches 30% of distance between the animal and vegetal pole
Gastrula period			
50%-epiboly	5 1/4		Blastoderm remains uniform in thickness
Germ-ring	5 2/3		Germ-ring visible from animal pole; 50%-epiboly
Shield	6	15	Embryonic shield visible from animal pole; 50%-epiboly
75%-epiboly	8	16	Dorsal side distinctly thicker; epiblast, hypoblast and evaculation zone visible
90%-epiboly	9		Brain rudiment distinctly thickened, notochord rudiment distinct from segmental plate
Bud	10	17	Tail bud prominent; notochord rudiment distinct from neural keel; early poster; midsagittal groove in anterior neural keel; 100% epiboly

Segmentation period			
1-somite	10 1/3		First somite furrow
5-somite	11 2/3	18	Optic vesicle appears; polster vesicle; Kupffer's vesicle present
14-somite	16	19	Embryo length (EL)= 0.9 mm; Otic placode; brain neuromeres, V-shaped trunk somites; yolk extension (YE) barely forming; NO: pronephric duct
20-somite	19	20[d]	EL=1.4 mm, muscular twitches; lens, otic vesicle, rhombic flexure; hindbrain neuromeres prominent; tail well extended
26-somite	22		EL= 1.6 mm; head-trunk angle (HTA)=125°; side-to-side flexures; otoliths; prim-3
Pharyngula period			
Prim-5	24		EL=1.9 mm; HTA=120°; otic vesicle length (OVL) = 5; early pigmentation in retina and skin; red blood cells on yolk, heart-beat
Prim-15	30		EL=2.5 mm; HTA=95°; OVL=3; retina pigmented; dorsal body pigment stripe; weak circulation; shallow pectoral fin buds; straight tail; early touch reflex; reduced spontaneous movements
Prim-25	36		EL=2.7 mm; HTA=75°; OVL=1; early motility; tail pigmentation and ventral body pigment stripe filling out; strong circulation; single aortic arch pair; pericardium not swollen
High-pec	42		EL=2.9 mm; HTA=55°; OVL<1; early lateral strip; complete dorsal strip; xanthophores in head only; iridophores in retina only; pericardium prominent; NO: heart chambers; segmental blood vessels; olfactory cilia
Hatching period			
Long-pec	48		EL=3.1; HTA=45°; resting dorsal up; dorsal and ventral strips meet at tail; iridophore plentiful on retina; distinct yellow cast to head
Pec-fin	60		EL=3.3 mm; HTA=35°; pectoral rudiment flattens into a fin with prominent circulation; iridophore retinal ring fills out; iridophore dorsal body stripe present; gut tract becoming visible
Protruding-mouth	72		EL=3.5 mm; HTA=25°; wide open mouth protruding anterior to the eye; iridophores in yolk stripe; eye half covered by iridophores; dorsal body as yellow as head

Note: [a] Hours after fertilisation at 28.5 °C.

[b] HB: Approximate stage number in the zebrafish stage series by Hisoaka and Battle (1958).

[c] Tiers: Horizontal rows of surface blastomeres, present in side view and counted between the margin and the animal pole.

d After HB stage 20, the HB descriptions are inaccurate.

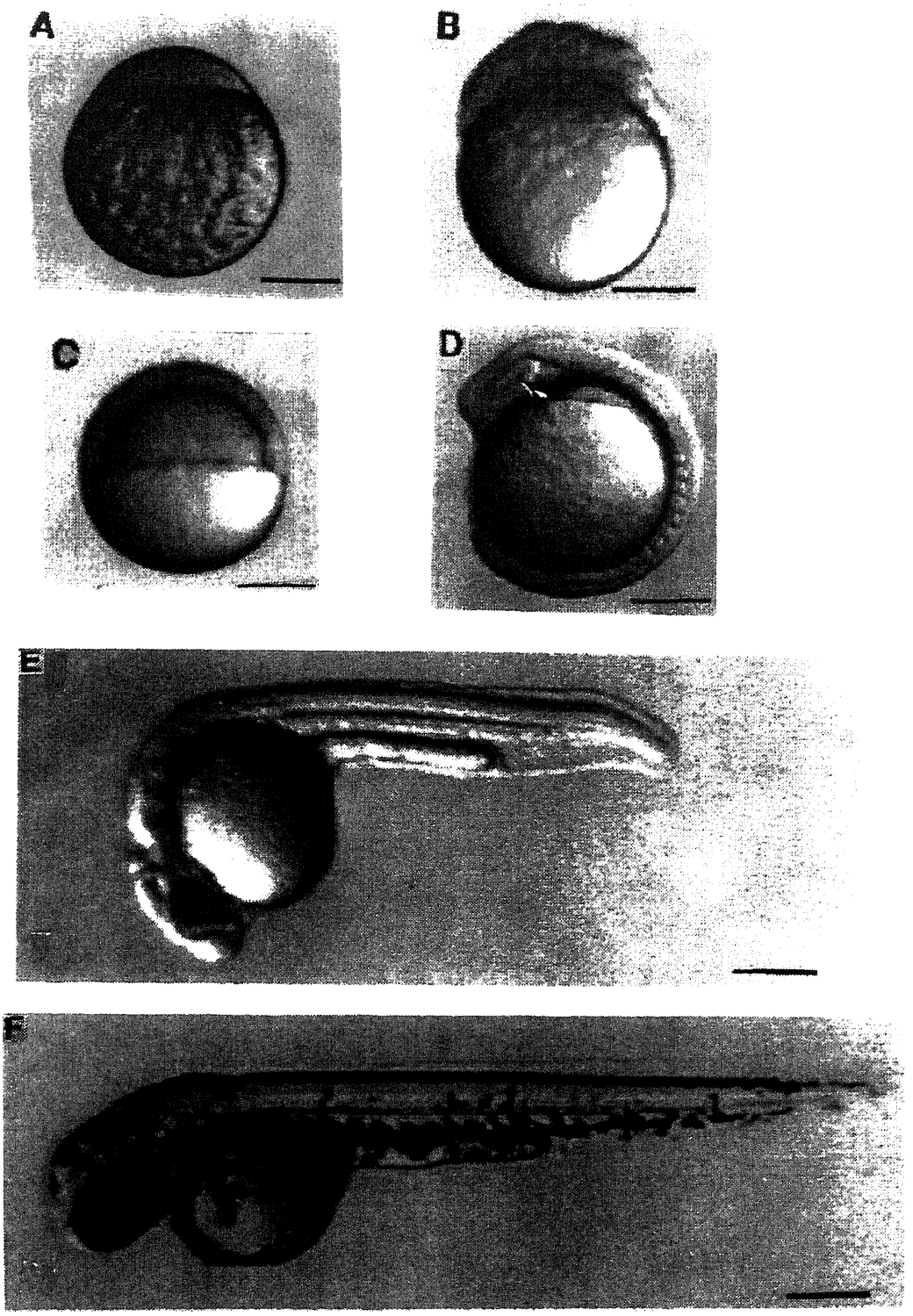


Fig. 1.3 Selected stages of zebrafish embryos during five embryo development periods at 28.5°C. **A**: 1-cell (zygote period); **B**: 64-cell (cleavage period); **C**: 50-%epiboly (gastrula period); **D** 8-somite (segmentation period); **E**: prim-5; and **F**: high-pec (Straightening period). Scale bar = 250 μm (from Kimmel *et al.*, 1995).

1.6 Intended approaches of the present study

The present investigation, using zebrafish embryos as a model, will include the following studies:

- **Feasibility study on vitrification of zebrafish embryos using methanol**

As high chilling sensitivity can be overcome by the vitrification approach (Steponkus *et al.*, 1990; Mazur *et al.*, 1992a), the low degree of cryoprotectant permeability of zebrafish embryos is the biggest challenge to achieving the successful vitrification. In an attempt to surmount this problem, a series of modifications to previous vitrification procedures for zebrafish embryos (Zhang and Rawson, 1996a) were proposed. The modified procedures consisted of utilising the most permeable embryo stages such as 1-cell (Zhang and Rawson, 1998), the most penetrating cryoprotectant of methanol (Zhang, 1994; Hagedorn *et al.*, 1996) and an ultra-rapid cooling rate, which has been proved to be effective in the vitrification of *Drosophila* embryos (Steponkus *et al.*, 1990; Mazur *et al.*, 1992) by mounting a single embryo on a gold electron microscope grid and plunging rapidly into LN_2 or N_2 slush.

- **Development of methods for the possible reduction of the high chilling sensitivity of zebrafish embryos**

If the chilling sensitivity of fish embryos could be reduced markedly, controlled rate slow cooling might be applied to fish embryo cryopreservation. In order to find ways to reduce the chilling injury in zebrafish embryos, several factors suspected to be associated with chilling injury in fish embryos, such as cooling rate, embryo development arrest by anoxia and partial removal of yolk, were investigated.

- **Investigation into the effect of partial removal of yolk on the cryobiological properties of zebrafish embryos**

The three major factors which are suspected to limit the cryopreservation of fish embryos are mainly related to the relatively large size of their yolk (Section 1.4). Using yolk-reduced embryos might alleviate the difficulties confronting fish embryo

cryopreservation. If a method could be developed for partial removal of yolk without major loss of viability, then it is proposed to investigate the cryobiological properties of the yolk-reduced embryos: the cryoprotectant toxicity; cryoprotectant permeability; and nucleation temperature of intaembryonic water. Hence the feasibility of utilising the yolk-reduced embryos for cryopreservation could be evaluated.

CHAPTER 2 MATERIALS AND METHODS

2.1 Introduction

Four main areas of study were covered by the project: a feasibility study of vitrification using methanol; effects of cooling rate and developmental arrest on the chilling sensitivity of zebrafish embryos; effect of partial removal of yolk on the chilling injury and cryoprotectant toxicity in zebrafish embryos; and a study on the characterisation of intraembryonic freezing and cryoprotectant penetration in zebrafish embryos using Differential Scanning Calorimetry (DSC). All the experimental work took place in The Research Centre, University of Luton except the DSC study which was carried out in the Jodrell Laboratory, Royal Botanic Gardens (Kew), Wakehurst Place.

2.2 General Methods

2.2.1 Maintenance of zebrafish (*Danio rerio*)

2.2.1.1 General information

Adult zebrafish (3 - 4 month old) were purchased from Beadals Aquatics, Luton, England. They were maintained in a 45 litre aquarium (28 × 28 × 58 cm) in an air-conditioned room at The Research Centre, University of Luton. Twenty to thirty fish were kept in each tank with an in-tank filtration system. Carbon-filtered tap water, which was aged in a 319 L tank (110 × 58 × 50 cm) for 4 - 7 days to release chlorine, was used and aerated constantly. The fish tanks were cleaned and over 80% of the water was changed weekly with complete renewal every 4 weeks. Other information for fish care is given in Table 2.1.

Aeration of the tank water in the tanks was carried out by using an electric pump to pump air under an upturned funnel that was surrounded by filter floss (King British®, England) in a plastic beaker (1 L) immersed in the fish tank. The funnel and floss were held in position by a layer of smooth gravel. Water was pulled though the gravel and floss by the suction effect generated by the rising air bubbles.

Table 2.1 General information for zebrafish care

Room temperature	26 ± 1°C
Water temperature	26 ± 2°C
Water pH	7.6 - 8.0
Water DO	80 ± 5 %
Water hardness express as $CaCO_3$	150 - 200 mg/l
Photoperiod	14 L : 10 D (Light : Dark)

2.2.1.2 Feeding

For normal maintenance, adult fish were fed twice a day with TetraMin flake fish food (ingredients: fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustaceans, oils and fats, algae, various sugars, EEC permitted colorants and preservative). For optimal mating conditions, fish were fed three times a day, with a supplement of live adult brine shrimp and/or daphnia juveniles in the middle of the day. Flake food was pulverized finely by hand. In order not to over feed the fish, the maximum amount of food that was fed to the fish at one time was only that amount that could be eaten within five minutes. After five minutes, excess food in the water was removed. During weekends or holidays, automatic fish feeders (Fish Mate F14 Aquarium Fish Feeder) were applied to feed fish.

2.2.1.3 Breeding and egg-collecting

Mature male and female fish (over 12-week-old) were separated for 5 - 10 days before breeding. Five to six females and 8 - 10 males were placed together in a mating tank during the late afternoon to early evening. A glass tray covered by a plastic net and plastic grass were placed into the tank to trap any eggs, which were heavier than water, that fell through the net away from the cannibalistic parents. The tray was removed from the tank in the morning and the eggs were collected, as spawning was induced by the first light of the morning. The newly fertilised embryos were washed in a fine steel mesh sieve and maintained at 26 ± 1°C in carbon-filtered, aerated tap water until they had developed the required stages.

2.2.1.4 Dechorionation of zebrafish embryos

Intact zebrafish embryos were dechorionated using enzymatic digestion since this method offers a practical means for rapid, simultaneous dechorionation of many fish eggs (Hallerman *et al.*, 1988). Embryos were treated with 2 mg/ml pronase (from *Streptomyces griseus*, Sigma Chemical Co.) made up in Embryo Medium (EM, a modified Hank's solution, Westerfield, 1995, Table 2.2) for 1-10 min at 26 ± 1°C with constant gentle shaking. The treatment time was dependent upon the developmental stage of the embryos and increases with increasing developmental stage. For instance, 2 min for 1-cell stage and 8 min for prim-6 stage. Embryos were then gently (especially with early stages) washed several times using EM to remove excess enzyme. Dechorionated embryos were cultured *in vitro* in EM until the desired stages were reached.

Table 2.2 Composition and preparation of embryo medium

Hank's Stock #1 (8.0 g NaCl and 0.4 g KCl in 100 ml deionised water)	1.0 ml
Hank's Stock #2 (0.358 g Na_2HPO_4 and 0.60 g KH_2PO_4 in 100 ml deionised H_2O)	0.1 ml
Hank's Stock #4 (0.72 g $CaCl_2$ in 50 ml deionised H_2O)	1.0 ml
Deionised H_2O	95.9 ml
Hank's Stock #5 (1.23 g $MgSO_4 \cdot 7H_2O$ in 50 ml deionised H_2O)	1.0 ml
Hank's Stock #6 (0.35 g $NaHCO_3$ in 10.0 ml deionised H_2O)	1.0 ml
1 M NaOH adjust PH to 7.2	

2.2.1.5 Determination of the developmental stage of zebrafish embryos

The developmental stage of a living embryo was determined by examination with a Leica DMIL microscope. During the segmentation period the elongated tail curves over the trunk and head so as to obscure the view if the embryo is left within its chorion, therefore dechorionated embryos were used for staging. Late in the pharyngula period, the dechorionated embryo was anesthetized with 0.003% tricaine (3-aminobenzoic acid ethyl ester methanesulfonate salt, Sigma Chemical Co.) at pH 7 (Westerfield, 1995) to prevent its movements in response to touch. Embryo stages were named (rather than numbered) based on corresponding morphological features described by Kimmel *et al.* (1995) (see Section 1.5.3).

2.2.2 Chemicals

Information on the chemicals used in this study is listed in Table 2.3. Fresh aqueous solutions were prepared in deionised water shortly before their use.

Table 2.3 Sources and purity of the chemicals used in this study

Compound	Source	Purity
NaCl	Aldrich Chemical Co. Ltd	99% minimum
KCl	Aldrich Chemical Co. Ltd	99% minimum
$CaCl_2$	Aldrich Chemical Co. Ltd	98% minimum
KH_2PO_4	Aldrich Chemical Co. Ltd	99% minimum
NaOH	BDH Limited Poole, England	98% minimum
$NaHCO_3$	BDH Limited Poole, England	99.5% minimum
Na_2HPO_4 (Anhydrous)	BDH Limited Poole, England	99.5% minimum
$MgSO_4 \cdot 7H_2O$	BDH Limited Poole, England	99.5% minimum
Propylene glycol (PG)	BDH Limited Poole, England	99.5% minimum
Sucrose	BDH Limited Poole, England	AnalaR
Methanol	SIGMA Chemical Co.	99% minimum
Dimethyl sulfoxide (DMSO)	SIGMA Chemical Co.	A.C.S. reagent

2.2.3 Embryo viability assessment

In preliminary studies, embryo viability was evaluated in terms of the percentage of treated embryos with normal developmental appearance following 3 day and 5 day culture. The normal development of the embryos was assessed using their morphological characteristics such as the formation of fins, tail, pericardium, mouth parts and swimbladder (for 5 day old embryos at 26°C) without malformations. As the survival of embryos at day 3 was found to be the same as that of a 5 day culture, embryo survival was normally only examined until the third day following treatments. For vitrification studies, morphological survival of the embryos was examined. Morphologically intact embryos were defined as those that possessed an intact membrane, and normal yolk and embryo structure.

2.2.4 Data analyses

2.2.4.1 Normalisation of embryo survival

Survival of untreated control embryos at different developmental stages, or sometimes even at the same stage, was variable from experiment to experiment, and survival of yolk-reduced embryos was significantly different from that of dechorionated embryos. Therefore, experimental survival was normalised with respect to the survival of the corresponding controls as follows:

$$\text{Normalised survival (\%)} = \frac{100}{\text{untreated control survival}} \times \text{experimental survival (\%)}$$

2.2.4.2 Replication and statistical analysis

The time-consuming nature of the research experimental work meant that there were limits to the number of replicates that could be used in different studies. For cryoprotectant toxicity tests and chilling sensitivity related studies, four to six measurements were made ($n = 4$ to 6), with a total of 80 to 180 embryos, for each treatment. For vitrification studies, at least 10 measurements were made ($n \geq 10$), and the total number of embryos used was in the range 90 to 120, for each regime. In DSC studies, three replicates were used ($n = 3$).

The overall effects of cryoprotectant, exposure period at low temperature (0 or -5°C) , developmental stage of embryos, cooling rate or anoxia on the survival of embryos in cryoprotectant toxicity or chilling sensitivity related studies, and the freezing temperatures of intraembryonic in DSC study were statistically analysed by using variance (ANOVA, one factor or two factors with replications). Student's *t*-test (two-tailed assuming unequal variances) was applied to determine differences in results between two contrasting groups such as the treated groups and the corresponding controls or two different treated groups. Values of P less than 0.05 were considered to be statistically significant. Means, standard errors, and threshold values for *t*-test and ANOVA were calculated using Excel (Version 7.0a for Windows, Microsoft Corp., USA). Where measurements are quoted numerically the standard error of the mean (SEM) is indicated by the '±' values. In graphs, SEM is shown by a bar.

2.2.5 Photomicrography of zebrafish embryos

A Leica DMIL microscope (with basic unit, focusing eyepiece, and type C-35 camera attachment) and photomicrographic camera (Ricoh XR-X 3PF) were used for photomicrography of zebrafish embryos. ISO 400/27° films (Kodak) were used for colour photomicrography.

2.3 Feasibility of Vitrification of Zebrafish Embryos Using Methanol

Intact zebrafish embryos at 1-cell, 64-cell, 50%-epiboly, 6-somite and prim-6 stages and dechorionated embryos at prim-6 stage were used in these experiments. Before vitrification studies, the sensitivity of 1-cell embryos to chilling at 0°C or 2 M methanol, and the lowest apparent vitrification concentrations of methanol, were determined. The effects of dilution, developmental stage of the embryos and dechorionation on the toxicity of 10 M methanol to embryos at 0°C were examined.

2.3.1 Sensitivity of intact 1-cell embryos to chilling or 2 M methanol

For chilling sensitivity tests, flat-bottomed test tubes containing 1-cell stage embryos in 2 ml EM were placed in wet ice (0°C) for 3, 5, 7, 10, 15, 20 or 30 min. After chilling, test tubes were placed into a 26°C water bath for 30 min. Embryos were then transferred to 100 ml beakers containing 50 ml EM and kept in the fish room (26 ± 1°C). Surviving embryos were checked and dead embryos were discarded daily until the fifth day. To evaluate 2 M methanol toxicity to 1-cell stage embryos, embryos were immersed in 2 M methanol for 10, 20, or 30 min at room temperature (23 ± 2°C). After equilibration, methanol was removed and the embryos were washed three times using EM and then incubated in the fish room for survival assessment (Section 2.2.3).

2.3.2 Determination of the lowest apparent vitrification concentrations (LAVCs) of methanol

A range of volumes (1, 3, 5, 8 and 10 μl) of methanol, at concentrations of 8, 10 or 12 M in EM, were loaded on gold electron microscope grids with a micro-pipette and rapidly cooled by plunging into either LN_2 (-196°C) or N_2 slush (a mixture of solid and liquid nitrogen, -205°C). N_2 slush was obtained by applying a vacuum pump to LN_2 for a few minutes. The LAVCs of methanol was defined as the lowest apparent concentration needed to vitrify both during cooling and warming. Ice formation was apparent by the whitening of the solution, and checked during both cooling and warming by direct visual inspection.

2.3.3 Toxicity of 10 M methanol at 0°C

Intact embryos at all five stages and dechorionated prim-6 embryos were used for the 10 M methanol toxicity study. In order to determine the optimal dilution method for removal of 10 M methanol, intact 6-somite embryos were transferred to test tubes containing 1 ml 10 M methanol at 0°C for 3, 5, 7, 10, 15, 20, 30 or 40 min, following a 30 min equilibration in 2 M methanol at room temperature. Two dilution methods were then investigated: a one-step EM dilution (10 ml EM was added to the test tubes and embryos were then washed in EM three times within 5 min); and a three-step sucrose dilution (1 ml 0.5 M sucrose was added to the test tubes and was then diluted 1:1 with EM twice, with 5 min for each step, before the embryos were washed thoroughly in EM). The optimal dilution method was used for the other four developmental embryo stages. The procedure used for toxicity testing of 10 M methanol to other stage embryos was the same as for 6-somite embryos, except for a shortened equilibration period in 2 M methanol at room temperature for one-cell (10 min) and 64-cell (20 min) stage embryos. The same procedure was also applied to dechorionated embryos at prim-6 stage to exam the effect of dechorionation on the 10 M methanol toxicity to the embryos. The subsequent assessment of survival was performed as described in Section 2.2.3.

2.3.4 Vitrification of zebrafish embryos

Based on the results obtained from the above experiments and previous studies (Zhang and Rawson, 1996a), two-step procedures (Table 2.4) were performed to pre-treat embryos at all stages with methanol before vitrification. Some embryos were exposed to 10 M

methanol at 0°C for up to 30 min so that methanol permeation and embryo dehydration were maximised. After 10 M methanol treatment at 0°C, embryos were individually loaded onto a gold electron microscope grid and dried using filter paper. The grid was plunged into LN_2 or N_2 slush and held for 30 seconds. The embryo was thawed in EM at room temperature and morphological survival was assessed (see Section 2.2.3) under a light microscope (WILD M32, HEERBRUGG, Switzerland) immediately and after a 20-minute culture in EM at room temperature.

Table 2.4. Two-step treatment procedures of zebrafish embryos before vitrification

Developmental stage of embryos	Step 1 (2 M methanol, 23±2°C)	Step 2 (10 M methanol, 0°C)
1-cell	10 min	1 min
64-cell	20 min	3 min
50%-epiboly	30 min	10 min
6-somite	30 min	5 min
prim-6	30 min	10 min

2.4 Effects of Cooling Rate and Developmental Arrest on the Chilling Injury of Zebrafish Embryos

Intact embryos at developmental stages of 64-cell, 50%-epiboly, 6-somite and prim-6 were used in these experiments. A controlled rate cooler (KRYO 10, Series II, Planer Products Ltd., England) was used to investigate the effect of cooling rate on the chilling injury of zebrafish embryos. Several different cooling rates: slow cooling, moderate rate cooling, and rapid cooling; and two chilling temperatures: 0°C and -5°C, were applied in these experiments. Anoxia was employed to arrest the development of intact embryos at 64-cell, 50%-epiboly and prim-6 stages, and the effect on embryo chilling injury was investigated.

2.4.1 Effect of different cooling rates on chilling injury in embryos

Twenty to thirty intact embryos were placed in Eppendorf tubes containing 1 ml EM, loaded in the chamber of the KRYO 10 controlled rate cooler, and cooled with a slow (0.3

or 1°C/min), or intermediate (30°C/min) cooling rate from a starting temperature of 20°C to either 0 or -5°C. The embryos were maintained at these final temperatures (0 or -5°C) for different periods of time (Table 2.5). Occasionally, the embryo medium froze during chilling at -5°C and these frozen samples were discarded. After chilling, the embryos were warmed immediately in a 26°C water bath, followed by 3 day culturing in EM at 26 ± 1°C for survival assessment (Section 2.2.3). For rapid cooling, embryos were mounted on gold electron microscope grids and then plunged into a pre-chilled beaker at 0 or -5°C. After being held at these temperatures, the embryos were warmed and survival assessed in the same way as those embryos cooled by a rate of 1°C/min or 30°C/min.

Table 2.5 Cooling procedures for four developmental stages of embryos

Developmental stage of embryos	Cooling rate (°C/min)	Chilling temperature (°C)	Exposure time (min)
64-cell	0.3, 1, 30, rapid *	0	10, 20, 30
	1, rapid	-5	1/6 **, 1
50%-epiboly	1, 30, rapid	0	60
	1, 30, rapid	-5	60, 1
6-somite	1, 30, rapid	0	60
	1, 30, rapid	-5	60, 1
Prim-6	1, 30, rapid	0, -5	60, 1

Note:* Rapid cooling was achieved by mounting embryos on gold electron microscope grids and then plunging them into a pre-chilled beaker at 0°C or -5°C.

** Only applied to rapid cooling.

2.4.2 Effect of anoxia on the development and survival of embryos

Intact embryos at 64-cell, 50%-epiboly, and prim-6 stages were placed in sealed 20 ml injection vials (Adelphi Ltd., England) with 5 ml EM. The rubber tops of the vials contained an inflow port connected to the high-purity pressure dry nitrogen source and an outflow port. Nitrogen gas was allowed to flow into the vials to replace the oxygen in the EM and the surrounding air. After 15 min nitrogen flushing, the ports were closed off and the embryos were held in anoxia condition for 4, and 24 h before they were transferred into normally aerated EM. The development of the embryos was assessed following the anoxia. Embryo survival was evaluated using the method described in Section 2.2.3.

2.4.3 Effect of developmental arrest on the chilling sensitivity of embryos

Chilling was initiated immediately after 15 min oxygen deprivation. Both anoxic and aerobic embryos at sphere, 50%-epiboly and prim-6 stages were held in sealed injection vials, and then located in the chamber of the controlled rate cooler. The embryos were cooled at 1°C/min from a starting temperature of 20°C to 0°C (for sphere stage) or -5°C (for 50%-epiboly and prim-6 stages). After holding for 10, 20 and 30 min (for sphere stage) or 1 h (for 50%-epiboly and prim-6 stages) at the final temperature, the embryos were warmed rapidly in a 26°C water bath, and then transferred into normally aerated EM for survival assessment.

2.5 Effect of Partial Removal of Yolk on the Chilling Injury and Cryoprotectant Toxicity in Zebrafish Embryos

Dechorionated zebrafish embryos at five developmental stages: 6-somite, 26-somite, prim-6, prim-15 and high-pec were used to evaluate the survival of embryos following partial removal of their yolk. Appropriate stages were chosen to investigate the effect of partial removal of yolk on the embryo sensitivity to chilling and cryoprotectant toxicity. Three cryoprotectants, methanol, DMSO and PG were tested in this study.

2.5.1 Removal of yolk from embryos

Dechorionated embryos at 6-somite, 26-somite, prim-6, prim-15 and high-pec stages were placed in an 8-well plastic dish and their yolk sac was punctured with a sharp micro-needle, made from a glass capillary, under a light microscope. Approximately 50 to 75% of yolk content was removed following multiple punctures. Those embryos in which part of yolk was removed were termed as 'yolk-reduced' embryos. In order to exam whether or not the mechanical wounding associated with the multi-punctures *per se* could affect the survival of embryos, dechorionated 6-somite and prim-6 embryos were cautiously punctured with a sharp micro-needle so that little yolk content was lost. The embryos which were punctured without any or with very little yolk loss were termed as 'punctured' embryos. The

yolk-reduced or punctured embryos were then washed and maintained in EM at 26 ± 1°C for survival assessment (Section 2.2.3).

2.5.2 Chilling sensitivity of yolk-reduced embryos

2.5.2.1 Effect of culture time on the chilling sensitivity of yolk-reduced embryos

Embryos at high-pec stage were used in these experiments. After 2, 6, or 24 h culture in EM following yolk removal, both control (dechorionated) and yolk-reduced embryos were loaded in flat-bottomed test tubes containing 5 ml EM. The test tubes were then immersed in iced water. After 6 h at 0°C, the test tubes were placed into a 26°C water bath for 30 min. Embryos were then transferred to 100 ml beakers containing 50 ml EM and held at 26 ± 1°C for 3 days for survival assessment. The survival of chilled embryos was normalised with respect to corresponding non-chilled controls maintained at 26 ± 1°C.

2.5.2.2 Effect of partial removal of yolk on the chilling sensitivity of embryos

Yolk-reduced embryos at both prim-6 and high-pec stages were used, after subsequent culturing for 24 h at 26 ± 1°C. Embryos were chilled at 0°C for 6 and 10 h before being returned to normal culturing conditions. Dechorionated control embryos were also chilled under the same conditions. All other procedures were the same as described above.

2.5.3 Effect of partial removal of yolk on cold shock of embryos

Both yolk-reduced and dechorionated control embryos at prim-6 were used in these experiments. After 24 h recovery at 26 ± 1°C following the removal of yolk, the embryos were cooled to -5°C by rapid cooling (as described in Section 2.4.1) and maintained at -5°C for 10, 30, and 60 min. A cooling rate of -1°C /min was also applied for comparison with an exposure period of 60 min at -5°C. All other procedures were the same as described above.

2.5.4 Toxicity of cryoprotectants to yolk-reduced embryos

2.5.4.1 Effect of cryoprotectants and exposure time

Dechorionated prim-6 embryos were punctured in 1 M methanol, DMSO or PG solution and more than half of the yolk content was released. The yolk-reduced embryos were transferred to test tubes containing 1 ml 1 M corresponding cryoprotectant solution. After 30, 60 or 120 min exposure at room temperature, 5 ml EM was added to each test tube to dilute the cryoprotectant solution. After 10 min, the embryos were washed several times with EM. The following culturing and survival assessment were the same as described above. Control (dechorionated) embryos were treated in the same way except that they were not punctured.

2.5.4.2 Effect of the concentration of methanol

Dechorionated prim-6 embryos were punctured in 1 M methanol and more than half of the yolk content was released before they were transferred to test tubes containing 1 ml 1, 2, or 3 M methanol, or 3 M methanol + 0.5 M sucrose solution for 30 min treatment at room temperature. The treated embryos were then diluted with 5 ml EM and washed thoroughly before culturing in EM at 26 ± 1°C for subsequent survival assessment.

2.5.5 Toxicity of cryoprotectants to punctured embryos

Dechorionated 6-somite embryos were cautiously punctured in 1 M methanol, PG or DMSO solution with a sharp micro-needle. Little yolk content was lost from the embryos during multi-punctures. The punctured embryos were exposed to 1 ml 1 M corresponding cryoprotectants in test tubes for 30 min at room temperature. The treated embryos were then diluted and washed several times with EM and maintained at 26 ± 1°C for subsequent survival assessment.

2.6 Characterisation of Intraembryonic Freezing and Cryoprotectant Penetration in Zebrafish Embryos

This study was carried out in the Jodrell Laboratory, Royal Botanic Gardens (Kew), Wakehurst Place. A DSC-7 (Perkin Elmer, USA) was employed to characterise intraembryonic freezing in zebrafish embryos. The operation of the DSC-7 is based on the power compensated "null-balance" principle (Block, 1994), in which energy evolved or absorbed by the sample and reference is compensated for by adding or subtracting the equivalent amount of electrical energy to a heater located in the sample holder. A continuous, automatic adjustment of heater power is necessary to maintain the sample holder temperature identical to that of reference, sample holder providing a varying electrical signal equivalent to the varying thermal behaviour of the sample. This measurement is made directly in energy units (milliwatts), and the power compensated DSC design allows for the direct measure of the energy of a transition. DSC measures the heat flow through a sample as it is scanned over a linearly changing temperature range or held at a single isothermal temperature.

The nucleation temperatures of intraembryonic water of zebrafish embryos were determined by the DSC study. The effect of embryo developmental stage, dechorionation, partial removal of yolk, cooling rate and the treatment of cryoprotectants on the temperatures of intraembryonic freezing were investigated. The amount of freezable water in single embryos was also calculated from the DSC measurements and the total water contents were determined gravimetrically.

2.6.1 Preparation of embryos

Both intact and dechorionated embryos at 6-somite, prim-6 and high-pec stages were used. Yolk-reduced high-pec embryos, which had been cultured for 24 h at 26 ± 1°C following partial removal of the yolk at prim-6 stage, were also tested. Dechorionated embryos at 6-somite or prim-6 stages were very fragile, and were therefore fixed with 3% gluteraldehyde (BDH Limited Poole, England) for 5 min at room temperature. Embryos were then washed several times with EM before DSC experiments.

The dechorionated embryos at 6-somite or prim-6 stage and yolk-reduced embryos at high-pec stage were used for the study of the effect of cryoprotectants on intraembryonic freezing. In order to examine the effect of multi-punctures on the permeability of embryos to cryoprotectants, punctured embryos at 6-somite and prim-6 stages were also used. Punctured embryos were produced as described in Section 2.4.1. Briefly, the yolk sac of the embryos was carefully punctured with a sharp micro-needle so that minimum yolk content was lost from the embryos. This is particularly important at the 6-somite stage since the removal of even a small part of the yolk would be lethal to the embryos. Dechorionated or yolk-reduced embryos were exposed to 2 M methanol or 1 M PG for 2 h at room temperature. For punctured embryos, the exposure time to either 2 M methanol or 1 M PG was 30 min at room temperature after a 2 h recovery period following multi-punctures. The cryoprotectant-treated embryos were then fixed with 3% glutaraldehyde for 5 min at room temperature and briefly washed several times with EM before DSC experiments.

2.6.2 Determination of the nucleation temperature of intraembryonic water

The DSC was calibrated using the melting of indium (melting point, 156.60°C; transition energy, 28.45 J/g) and the crystalline transition of cyclohexane (-87.06°C). The thermal lag of the calorimeter was checked using the melting point of double-deionised water. The DSC was first allowed to reach thermal equilibrium at 25°C. A standard aluminum pan with a pan lid was weighed on an ultramicrobalance (Sartorius 4504 MP-1, accuracy = ± 0.1μg). One to three intact or dechorionated zebrafish embryos were transferred to the bottom of the sample pan. The embryos were separated from each other and the surface water around the embryos was removed by blotting with a piece of filter paper. In order to minimise dehydration during the DSC experiment and also to displace possible residual extraembryonic water, 2 μl of silicon oil (n_D^{20} 15,040, Aldrich Chemical Co. Ltd) was added on to each embryo. The pan lid was put in place and sealed using a crimper. The pan (with the lid and the sample) was weighed again and then loaded into the DSC. A similar empty pan was used to serve as a reference. The experimental procedure was as follows:

a) Cool from 25 to -40°C at 10°C/min;

b) Hold at -40°C for 1 min (to allow the sample to reach thermal equilibrium);

c) Warm from -40° to 25°C at 10°C/min.

Some experiments were performed using a 2°C/min cooling/warming rate to determine whether or not cooling rate affect the nucleation temperatures of the embryos.

Thermogram data were analysed using the Perkin Elmer Pyris Thermal Analysis software (Version 3.52). The effects of the developmental stage of embryos, dechorionation, partial removal of yolk, cooling rate and the exposure to cryoprotectants on the nucleation temperatures were studied.

2.6.3 Determination of intraembryonic water content

2.6.3.1 Freezable water content

The amount of freezable water was calculated from the heat energy that was absorbed during freezing. The freezing enthalpy (ΔH_{freeze}) of the water crystallisation was determined by the integrated heat flow over the temperature range of the transitions and obtained by analysing the thermogram using the Pyris Thermal Analysis software. To determine ice content with reference to the freezing enthalpy (ΔH_{freeze}), corrected values of heat fusion (L_f) were used because of the decrease of L_f with the reduction of temperatures (see Chapter 6). The amount of water frozen was calculated as $\Delta H_{freeze}/ L_f$.

2.6.3.2 Total water content of zebrafish embryos

Dechorionated embryos at 6-somite, prim-6 and high-pec stages and yolk-reduced embryos at high-pec stage were used. To calculate the average total amount of water in an embryo, ten embryos of each stage were placed on a pre-weighed aluminum pan (W_1). Embryos were blotted using filter paper and further surface water was removed by applying a hair-dryer for a few seconds. The pan with the embryos was re-weighed (W_2), then placed in a 103°C oven, dried for 6 h and weighed again (W_3). The total water content per embryo was determined as (W_2 - W_3)/10. The fully hydrated weight per embryo was also calculated as (W_2 - W_1)/10. All weights were determined using the ultramicrobalance mentioned above.

CHAPTER 3 FEASIBILITY STUDY ON VITRIFICATION OF ZEBRAFISH EMBRYOS USING METHANOL

3.1 Introduction

Previous attempts at the cryopreservation of zebrafish embryos by vitrification failed to preserve embryo viability (Zhang and Rawson, 1996a), with the main problem being identified as the low permeability of embryo membranes at the stages tested (6-somite and heart-beat) to cryprotectants. The YSL has been reported by other workers (Hagedorn *et al.*, 1996) to play a critical role in limiting the permeation of some cryoprotectants throughout zebrafish embryos. As the YSL begins to form at 512-cell stage (2.5-3 h after fertilisation) during blastula (Kimmel *et al.*, 1995), embryos at earlier stages should have higher permeability to cryoprotectants if the YSL is the main permeability barrier. Recently, the permeability of one-cell stage embryos to cryoprotectant methanol has been found to be over ten times higher than that of later developmental stages (Zhang and Rawson, 1998). Although zebrafish embryos at early stages are much more sensitive to chilling (Zhang and Rawson, 1995; Hagedorn *et al.*, 1997b) and cryoprotectant toxicity (Adam *et al.*, 1995; Zhang and Rawson, 1993), the exposure time to cryoprotectants could be kept to a minimum as their membranes are more permeable to cryoprotectants, and vitrification would help to overcome the problems associated with the high chilling sensitivity of the early stage embryos (Steponkus *et al.*, 1990). Therefore, embryos at early developmental stages of 1-cell and 64-cell were used in the present study. Other stage (50%-epiboly, 6-somite and prim-6) embryos were also tested for comparison.

Methanol penetrates mammalian cells and embryos very rapidly (Wright and Diamond, 1969; Naccache and Sha'afi, 1973; Rall *et al.*, 1984) and is an effective cryoprotectant in both slow-cooling (Czlonkowska *et al.*, 1991; Rall *et al.*, 1984; Rapatz, 1973) and vitrification (James, 1980). Moreover, several workers have suggested that methanol is the only cryoprotectant to permeate zebrafish embryos quickly (Zhang and Rawson, 1996a; Hagedorn *et al.*, 1996), and its toxicity to these embryos is also low (Zhang *et al.*, 1993). Methanol was therefore chosen as the cryoprotectant for this vitrification study.

In order to optimise the pre-treatment procedures of vitrification, experiments were carried out to investigate the sensitivity of 1-cell stage embryos to 2 M methanol, chilling, and the toxicity of methanol at a vitrifying concentration to all embryo stages prior to vitrification studies. The vitrification concentrations of methanol are inversely related to the cooling rate. Increasing the cooling rate lowers the vitrification concentration of methanol and thus minimises the toxicity. The most rapid cooling rate avaivable under our laboratory conditions was achieved by mounting a single embryo on a gold electron microscope grid and plunging it into LN_2 or N_2 slush, a technique which had proven to be effective in the vitrification of *Drosophila* embryos (Steponkus *et al.*, 1990; Mazur *et al.*,1992).

Intact embryos were used in this study as the chorion is considered to be freely permeable to water and the low molecular weight cryoprotectant methanol (Zhang and Rawson, 1996a), and also dechorionated embryos at early stages are extremely sensitive to exposure to increased surface tensions which are inevitable in the vitrification procedures applied here. Only dechorionted embryos at prim-6 stage, which are able to resist the increased surface tensions, were tested for comparison.

3.2 Results

3.2.1 Sensitivity of one-cell embryos to chilling and 2 M methanol

Fig. 3.1 shows 1-cell stage embryos to be very sensitive to chilling. After only 5 min exposure at 0°C, embryo survival decreased significantly ($p < 0.01$) when compared with room temperature controls. Approximately 50% of the embryos did not survive exposure to 0°C for 10 min and all embryos succumbed after 30 min at 0°C. Therefore, the exposure time at 0°C was limited to 10 min in subsequent experiments with 1-cell embryos.

The toxicity studies showed that 1-cell embryos can tolerate 2 M methanol for 10 min at room temperature with normalised survival of 85.1 ± 2.9%. However, 2 M methanol was toxic ($p < 0.01$) to 1-cell embryos when equilibration time was extended to 20 min with embryo survival of only 31.9 ± 3.6% and all the embryos were killed after a 30 min equilibration. Based on these results, the exposure time for 1-cell embryos to 2 M methanol was shortened to 10 min prior to transfer to 10 M methanol at 0°C.

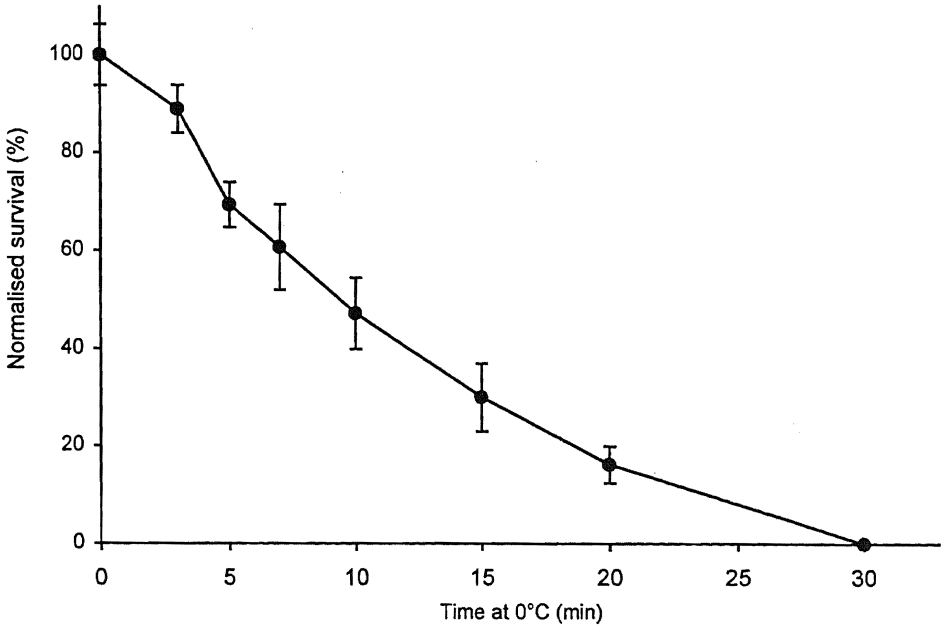


Fig. 3.1 Chilling sensitivity of 1-cell embryos of zebrafish at 0°C. Embryo survival was normalised with respect to that of room temperature controls, which averaged 62.7 ± 6.3%. Values are means ± SEM (n = 6).

3.2.2 Determination of lowest apparent vitrification concentrations of methanol

As shown in Table 3.1, 8 M methanol did not vitrify in LN_2 even when the smallest sample volume (1 μl) was applied. However, vitrification did occur during cooling when N_2 slush was used if the sample volume did not exceed 8 μl, although ice formation was inevitable during warming. The results also showed that during cooling 10 M methanol vitrified in LN_2 when the volume was smaller than 10 μl and vitrified in N_2 slush for all the applied volumes, but during thawing, only 1 μl volume remained vitreous and devitrification occurred in all other cases with both cryogenic fluids (LN_2 and N_2 slush). When the concentration of methanol was increased to 12 M, vitrification was achieved during cooling and maintained during warming even with the largest volume tested (10 μl). The results showed that the LAVCs of methanol for 1 μl and 10 μl sample volume were 10 M and 12 M respectively.

Table 3.1 Vitrification and devitrification features of methanol at several concentrations under different volumes of sample and cryogenic fluids

Cryogenic fluids		Liquid nitrogen					Nitrogen slush				
Volume of sample (μl)		1	3	5	8	10	1	3	5	8	10
8 M	cooling	C	C				V	V	V	C	C
8 M	warming	C	C				DV	DV	DV	C	C
10 M	cooling	V	V	V	V	C	V	V	V	V	V
10 M	warming	V	DV	DV	DV	DV	V	DV	DV	DV	DV
12 M	cooling				V	V				V	V
12 M	warming				V	V				V	V

Note: C: Crystallisation resulted in whitening of the solution.
V: Vitrification was evidenced by the formation of a transparent glass.
DV: Devitrification conferred the occurrence of whitening rather than remaining vitreous during warming.

3.2.3 Toxicity of 10 M methanol at 0°C

3.2.3.1 Effect of dilution

Comparisons of the survival of 6-somite stage embryos using two different dilution methods for the removal of cryoprotectant after 10 M methanol treatment at 0°C showed that one-step EM dilution was significantly superior ($p < 0.01$) to three-step sucrose dilution although the significance was less pronounced with longer (> 15 min) exposure period (Fig. 3.2). One-step EM dilution was therefore applied to other stage embryos.

3.2.3.2 Effect of developmental stages

Early stage (1-cell and 64-cell) embryos showed higher ($p < 0.01$) sensitivity to 10 M methanol at 0°C than later stages (50%-epiboly to prim-6) (Fig. 3.3). Embryo survival dropped significantly ($p < 0.01$) even after the shortest treatment period (3 min), being 17.6 ± 5.5% and 79.3 ± 11.2% for 1-cell and 64-cell respectively. No 1-cell embryo survived after 7 min treatment, whereas no significant reductions were observed in survival after 10,

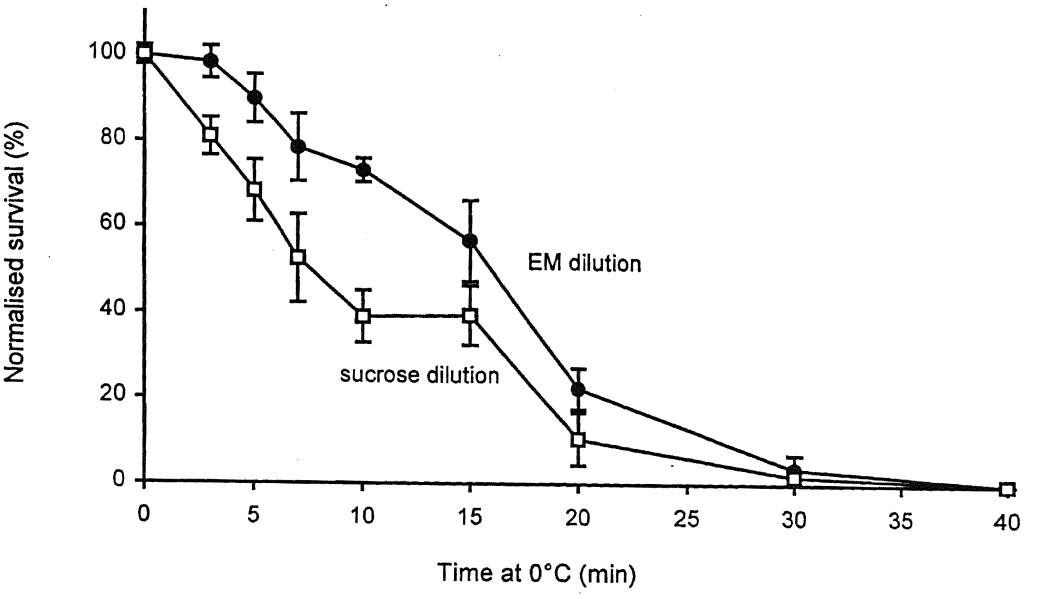


Fig. 3.2 Effect of dilution on the toxicity of 10 M methanol at 0°C to 6-somite embryos. Embryo survival was normalised with respect to room temperature control, which averaged 95.1 ± 1.2%. Values are means ± SEM (n = 4).

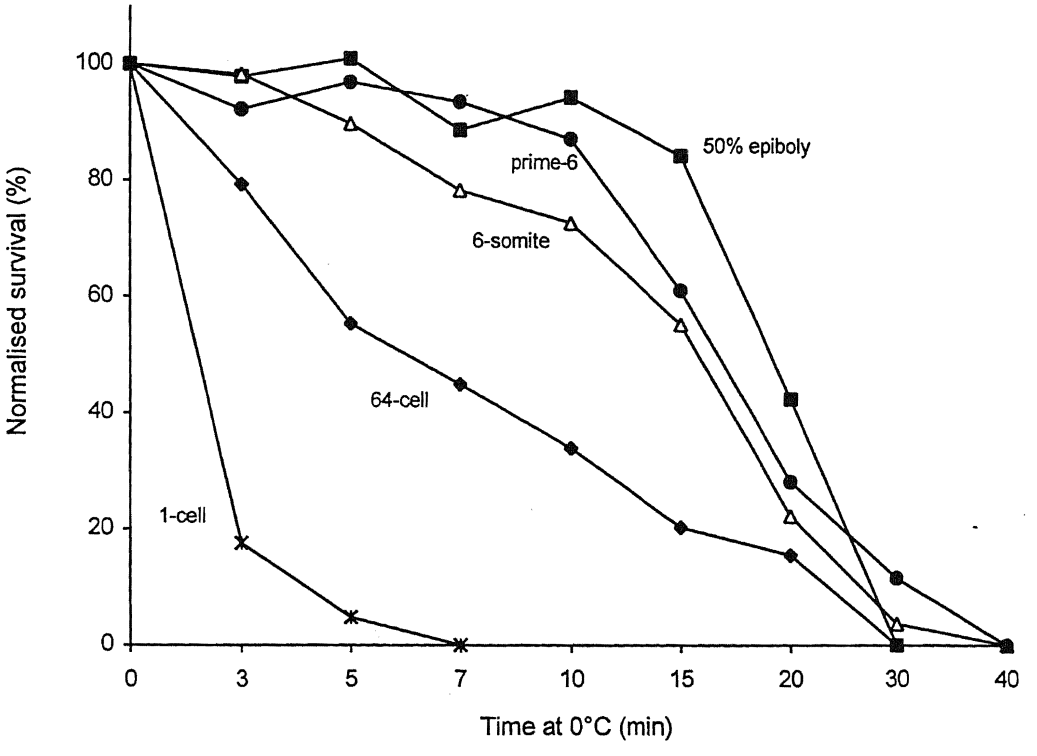


Fig. 3.3 Toxicity of 10 M methanol to zebrafish embryos at five developmental stages after exposure for up to 40 min at 0°C. Embryo survival was normalised to room temperature control, which averaged 85.1 ± 6.6% (1-cell), 76.5 ± 9.8% (64-cell), 95.1 ± 2.9% (50%-epiboly), 95.2 ±1.2% (6-somite) and 97.6 ± 1.8% (prim-6). Values are means ± SEM (n = 4). The error bars of SEM are not shown in the graph. The values of SEM are in the range of 2.1 to 10.8.

5 or 10 min exposure for 50%-epiboly, 6-somite, and prim-6 stage embryos respectively. Considering all the exposure times tested, no significant difference ($p > 0.05$) was observed among the three later stages. Embryos at all stages were killed after 40 min treatment of 10 M methanol at 0°C. According to these results, the exposure of embryos to 10 M methanol prior to vitrification was limited to 1 min for 1-cell, 2 min for 64-cell, 5 min for 6-somite and 10 min for 50%-epiboly and prim-6 stages.

3.2.3.3 Effect of dechorionation

The toxicity of 10 M methanol on dechorionated prim-6 embryos appeared to be manifest more quickly than that on intact embryos (Fig. 3.4). The survival of the dechorionated embryos was reduced significantly ($p < 0.05$) after just 7 min exposure to 10 M methanol at 0°C, whereas no significant ($p > 0.05$) survival reduction was observed with intact embryos within 10 min exposure. However, all of the dechorionated and intact embryos were killed after 40 min exposure to 10 M methanol at 0°C.

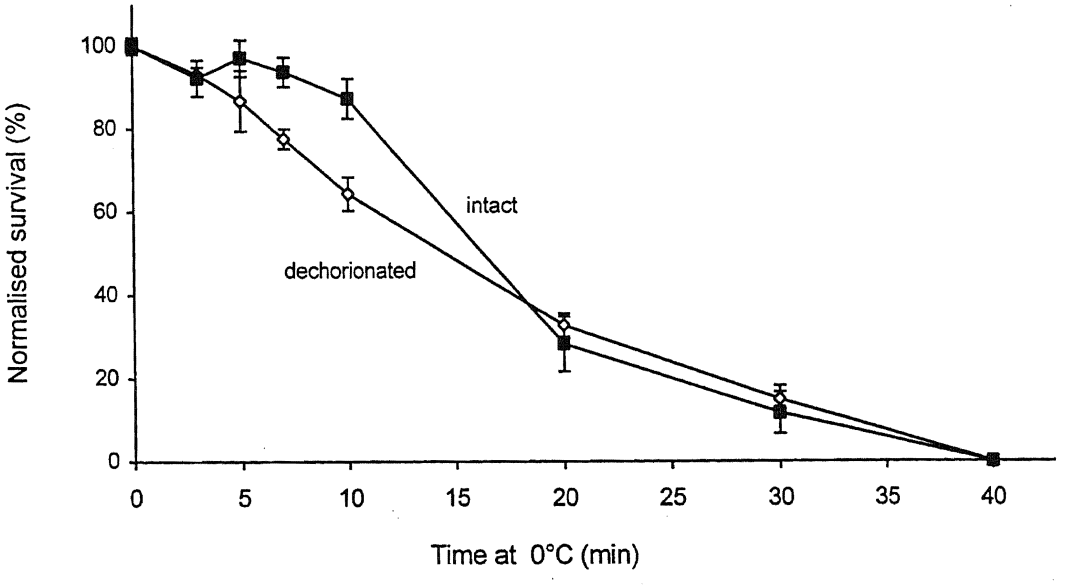


Fig. 3.4 Comparison of survival of dechorionated and intact embryos at prim-6 stage after 3 to 40 min exposure to 10 M methanol at 0°C. Embryo survival was normalised with respect to room temperature control, which averaged 76.7 ± 1.5% and 97.6 ± 0.9% for dechorionated and intact embryos respectively. Values are means ± SEM (n = 4).

3.2.4 Vitrification of zebrafish embryos

Following vitrification, approximately 50% of early stage (1-cell and 64-cell) embryos and 80% of later stage (50%-epiboly to prim-6) embryos remained morphologically intact as observed by light microscope inspection immediately after thawing. However, collapse of the plasma membrane and rupture of the yolk of embryos at all stages were observed within 20 min of thawing. All embryos were found to become opaque during cooling, and temporarily whiten during warming. These observations also applied to those embryos which remained morphologically intact and transparent even after the longest exposure periods to 10 M methanol at 0°C before vitrification. No embryos showed any developmental ability after vitrification. N_2 slush did not significantly improve survival when compared with LN_2. Similar results were also obtained from vitrification of dechorionated embryos at prim-6 stage.

3.3 Discussion

3.3.1 Sensitivity of one-cell embryos to chilling and 2 M methanol

The results from embryo chilling sensitivity studies are in agreement with the previous findings that zebrafish embryos at early stages (1.25 to 3 h) were more chill-sensitive than late stages (50%-epiboly to heart-beat) (Zhang and Rawson, 1995). Hagedorn *et al.* (1997a) reported less than 40% early stage embryos (1.25 to 2 hr) survived after 10 min exposure to 0°C, and in this present study, about 50% 1-cell embryos survived under the same condition. The abundant amounts of intraembryonic lipids in the early stage embryos (Heming and Buddington, 1988) are possibly responsible for the high chilling sensitivity of embryos. The reduction of chilling sensitivity and successful cryopreservation of early stage porcine embryos after removal of their intraembryonic lipids may confirm such an explanation, at least for porcine embryos (Nagashima *et al.*, 1994, 1995).

Embryos at the 1-cell stage also showed high sensitivity to 2 M methanol. They did not tolerate 2 M methanol for more than 10 min at room temperature, and no embryo survived 30 min exposure, whereas according to previous reports, embryo survival of later stages

(6-somite and heart-beat) was not decreased after 30 min exposure to 2 M methanol at room temperature (Zhang and Rawson, 1996a). Adam *et al.* (1995) investigated the overall enzymatic activity of lactate dehydrogenase and glucose-6-phosphate in zebrafish embryos after equilibration in the cryoprotectant DMSO or ethylene glycol and also found that early cleavage stage is the least resistant to cryoprotectants. This is probably associated with the relatively high membrane permeability to cryoprotectant at early stages (Czlonkowska *et al.*, 1991), suggesting that a shortened equilibration time in 2 M methanol should be applied for the 1-cell stage embryos before vitrification.

3.3.2 Lowest apparent vitrification concentrations of methanol

The LAVCs of cryoprotectants are closely related to their physical-chemical properties, hydrostatic pressure, cooling/warming rates (Fahy *et al.*, 1984), sample size, sample carriers, cryogenic fluids and warming methods (Fahy *et al.*, 1990a; Han *et al.*, 1995; Mazur *et al.*, 1993; Rall and Fahy, 1985). N_2 slush is able to prevent the formation of a thermal insulation layer between cryogenic fluid and the sample and thus to increase the rate of cooling (Crowley *et al.*, 1961; Han *et al.*, 1995). The results of the effects of cryogenic fluids and the volume of the sample on the LAVCs of methanol showed that N_2 slush helped methanol to vitrify during cooling, but made little contribution to the inhibition of devitrification during warming. At a fixed concentration of methanol, the maximum volume of sample which could be thawed without devitrification was much smaller than that which could be vitrified. The LAVCs, defined as the lowest concentration needed to vitrify during both cooling and warming, were 10 M and 12 M for 1 µl and 10 µl respectively under the condition applied in this study. As the volume of an intact zebrafish embryo is less than 1 µl (about 0.5 µl), a concentration of 10 M methanol was chosen for toxicity assessment before the vitrification study.

3.3.3 Effect of dilution method, embryo stage and dechorionation on the toxicity of 10 M methanol

Introduction and removal of cryoprotectant are two of the many factors that have been demonstrated to affect successful cryopreservation. Based on the previous findings that 2 M methanol permeated the embryos within 15 min (Hagedorn *et al.*, 1996) and was not shown

to be toxic to late stage (later than 50%-epiboly) embryos exposed for 30 min at room temperature (Zhang *et al.*, 1993), later stage embryos were pre-treated with 2 M methanol for 30 min before introducing 10 M methanol for further penetration and dehydration. In order to reduce the toxicity of methanol, exposure to 10 M methanol was performed at 0°C rather than room temperature. Two approaches for removal of cryoprotectant were compared. A three-step sucrose dilution method, which slowed the rate of cryoprotectant removal and therefore reduced the possible injury arising from osmotic shock; and a one-step EM dilution, which was used to speed the removal of cryoprotectant so that the toxicity of methanol could be minimised (Fahy *et al.*, 1984). The one-step EM dilution resulted in higher embryo survival than three-step sucrose dilution, indicating that the intrinsic toxicity of methanol rather than osmotic stress was responsible for the reduction of embryo survival. These findings are similar to those obtained by Fahy (1984) for DMSO and acetamide toxicity with mammalian embryos, and Lepopold and Atkinson (1999) for butane-2,3-diol with insect *Lucilia cuprina* embryos. Later stage embryos were much more tolerant to 10 M methanol than early stages and this is probably related to the progressive decrease in the permeability of the plasma membrane to cryoprotectant (Alderdice, 1987; Potts and Eddy, 1973; Zhang and Rawson, 1998). Dechorionated embryos have much reduced volumes, are free of an outer barrier, and would appear to have clear advantages for use in vitrification if they were able to resist the surface tension during vitrification procedures and not be less tolerant to cryoprotectants. However, the results showed that the dechorionated embryos at prim-6 stage appeared to be more sensitive to 10 M methanol at 0°C compared with intact embryos.

3.3.4 Vitrification of embryos

In the present study, approximately 80% late stage embryos remained morphologically intact after vitrification, compared with the results of previous report (Zhang and Rawson, 1996a) stating only 32% obtained for the same stage (6-somite) after vitrification in straws. indicating that the vitrification procedures applied in this study have advantages over previous methods. Steponkus *et al.* (1990) reported the first successful case of cryopreservation of *Drosophila* embryos by using copper grids and N_2 slush in vitrification, although no embryos survived using polypropylene straws as specimen containers in the same procedure. The ultra-rapid cooling and warming rates arising from this method probably account for the improvements. Although zebrafish embryos at early stage such as

1-cell have been reported to be more permeable to methanol (Zhang and Rawson, 1998), the inevitable intraembrynoic ice formation of the embryos during cooling showed that embryo dehydration and methanol permeation were not sufficient to reach vitrifying conditions. The higher sensitivity of early stage embryos to chilling and methanol might account for their lower morphological survival than that seen in later stage (50%-epiboly to prim-6) embryos following vitrification. Intraembryonic freezing could not be avoided even with those embryos which had been exposed to 10 M methanol for 30 min at 0°C and still appeared to be transparent. The same results were obtained from the experiments with N_2 slush and dechorionated embryos, indicating that even the most rapid cooling rate under our laboratory conditions was still not fast enough to vitrify the embryos.

3.4 Summary

This study investigated the feasibility of vitrification of zebrafish embryos using methanol as the cryoprotectant. The lowest apparent vitrification concentration of methanol was found to be 10 M for 1 μl volume. After 10 M methanol exposure at 0°C, a one-step EM dilution method for the removal of the cryoprotectant resulted in higher embryo survival when compared with a three-step sucrose dilution method. The toxicity of 10 M methanol was much more pronounced with early stage (1-cell and 64-cell) embryos than later stages (50%-epiboly, 6-somite and prim-6). Embryos at both early stages did not withstand 3 min exposure to 10 M methanol at 0°C, and the maximum time periods for 50%-epiboly, 6-somite and prim-6 stage embryos to tolerate 10 M methanol without significant detrimental effects were 10, 5, and 10 min respectively. No embryo survived after 40 min exposure. Compared with previous studies, vitrification of embryos on gold electron microscope grids improved their morphological survival, being ~50% and 80% for early and late stage embryos respectively. However, no embryos showed any developmental ability, and the application of nitrogen slush and dechorionated embryos did not improve the results of vitrification.

Unsuccessful vitrification of zebrafish embryos in this study, employing the most permeable embryo stage (1-cell), most penetrating cryoprotectant (methanol) and ultra-rapid cooling rate, strongly suggested that the low permeability of zebrafish embryos to cryoprotectant and water remains the biggest problem. Approaches to overcoming the low permeability of zebrafish embryos need to be developed before effective vitrification

procedures can be designed. An alternative approach for achieving successful zebrafish embryo cryopreservation is to reduce their high chilling sensitivity sufficiently so that controlled slow cooling can be applied to their cryopreservation. However, the nature of the chilling injury in these embryos needs to be determined before methods for reducing their high chilling sensitivity can be found.

CHAPTER 4 EFFECTS OF COOLING RATES AND DEVELOPMENTAL ARREST ON THE CHILLING INJURY IN ZEBRAFISH EMBRYOS

4.1 Introduction

High chilling sensitivity of zebrafish embryos is one of the main obstacles to their successful cryopreservation (Zhang and Rawson, 1995). In order to reduce the chilling sensitivity of zebrafish embryos and increase the chance for successful cryopreservation, it is essential to understand the basis and exact nature of the chilling injury.

Chilling injury is seen in many tissues and cell-types following exposure to low temperatures without freezing, and is usually classified into two distinct categories (Morris and Watson, 1984): direct chilling injury or cold shock and indirect chilling injury (Section 1.2.1). Although a wide variety of cell-types are observed to be sensitive to cold shock, the clearest example of cold shock is probably the response of spermatozoa to chilling since the effects on motility appear very quickly and depend mainly on cooling rate (Watson, 1981; Drobnis *et al.*, 1993). Studies with *Drosophila* embryos showed that chilling injury is a function of the exposure time at low temperatures and independent of cooling rate, suggesting an indirect chilling injury (Mazur *et al.*, 1992). In some cases, such as bovine oocytes, it seems difficult to determine whether the effect is due to cold shock or to indirect chilling injury because the chilling injury develops very rapidly (Martino *et al.*, 1996). It is generally agreed that it is the thermotropic behavior of membrane lipids which is the factor determining cold shock (Morris, 1987), and lipid phase transitions in cell membranes has been confirmed to be responsible for cold shock injury in a range of sperm (Holt *et al.*, 1988; Drobnis *et al.*, 1993), bovine oocytes (Arav *et al.*, 1996), and human platelets (Crowe *et al.*, 1999). One possible mechanism of indirect chilling injury proposed by Mazur *et al.* (1992) is that lowered temperatures result in the accumulation of defective enzymatic products due to the loss of necessary synchrony of coupled reactions involved in embryological development. However, indirect chilling injury may also be associated with lipid phase transition, but the mechanism seems to be more complicated as a reduction in temperature may affect the structure and activity of both proteins and lipids, and the resulting effects on cell physiology are bewildering in complexity (Morris and Clarke, 1987).

Previous studies (Zhang and Rawson, 1995) showed that chilling sensitivity of zebrafish embryos is stage-dependent and survival of embryos decreased with storage time and temperature. However, no published data are currently available on whether or not the chilling injury of zebrafish embryos is associated with the rate of cooling, or whether the embryos are susceptible to cold shock injury arising from rapid cooling. This study is intended to help fill these information gaps. Several different cooling rates: slow cooling (0.3°C/min or 1°C/min), moderate rate cooling (30°C/min) and rapid cooling; two chilling temperature: 0°C and -5°C; and four developmental stages: 64-cell, 50%-epiboly, 6-somite and prim-6 were applied to investigate the effect of cooling rate on the chilling sensitivity of zebrafish embryos. The study is also designed to determine if the indirect chilling injury in zebrafish embryos is associated with high embryo development rate, by applying anoxia to arrest reversibly the development of embryos. Embryos were oxygen-deprived by flowing nitrogen gas into the embryo medium, and the effect of anoxia on embryo development, viability, and indirect chilling injury were evaluated.

4.2 Results

4.2.1 Effect of cooling rate on chilling injury in zebrafish embryos

4.2.1.1 Effect of exposure time and temperature on chilling injury in 64-cell embryos

The effect of cooling rate on the survival of 64-cell embryos after different exposure times at 0°C is shown in Fig. 4.1. There was a significant effect ($p < 0.05$) of cooling rate and exposure time on embryo survival. When the exposure period was 10 or 20 min, rapid cooling resulted in higher survival compared with slower cooling rates. However, after 30 min exposure at 0°C, no embryos survived no matter what cooling rate was. When the cooling rate was 0.3°C/min, all embryos succumbed within 10 min exposure at 0°C. The results indicated that 64-cell stage embryos are more sensitive to chilling over longer exposure period than to cold shock resulting from rapid cooling.

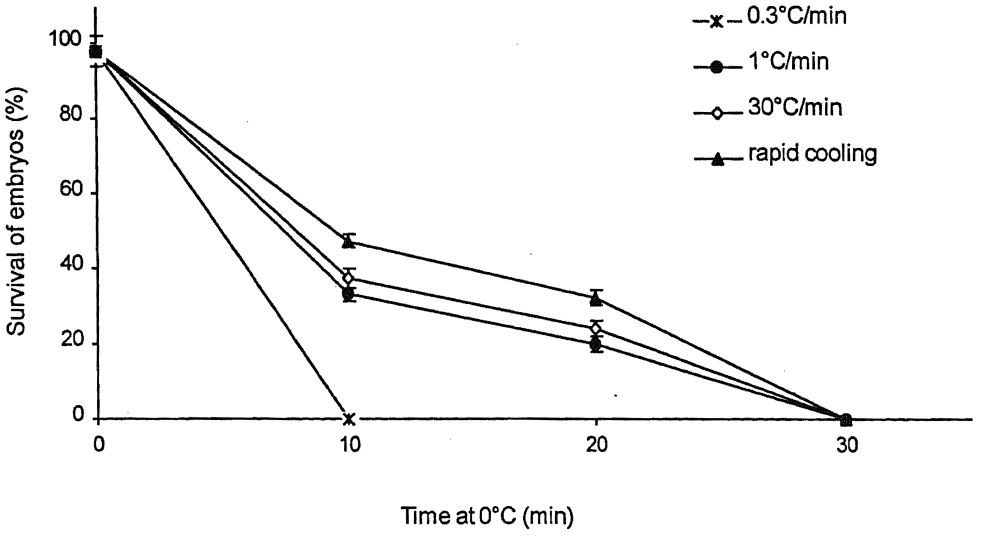


Fig. 4.1 Effect of cooling rate on the survival of 64-cell embryos after 10, 20 and 30 min exposure to 0°C. The survival of non-chilled control embryos was 98.3 ± 1.1 %. Values are means ± SEM (n = 6).

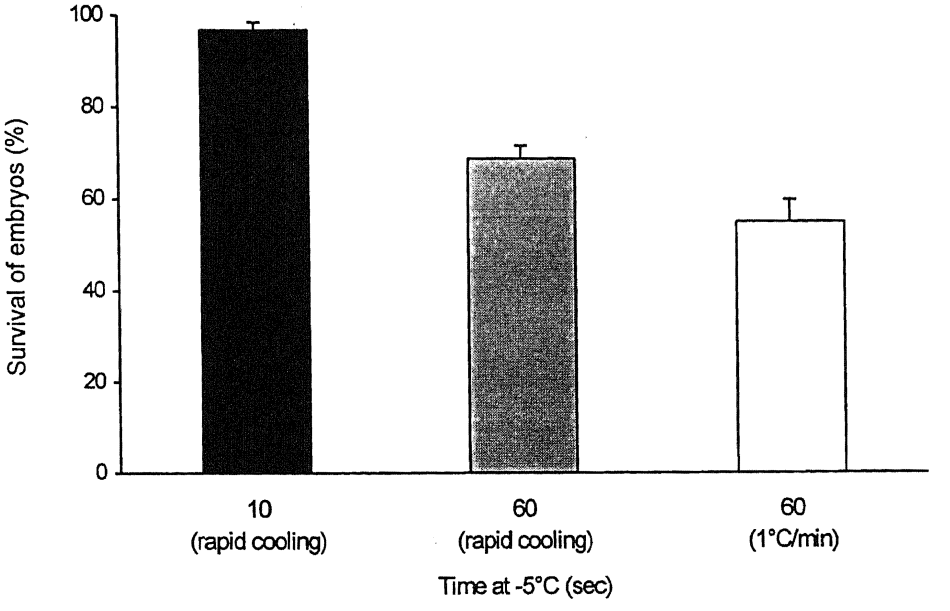


Fig. 4.2 Effect of exposure time at - 5°C on survival of 64-cell embryos after both rapid and slow cooling (1°C /min). The survival of non-chilled control embryos was 95.8 ± 2.4%. Values are means ± SEM (n=6).

When the 64-cell embryos were cooled rapidly to -5°C (Fig. 4.2), just 1 min chilling resulted in significant reduction ($p < 0.01$) in embryo survival whilst a rapid 10 second exposure to -5°C did not cause ($p > 0.05$) such reduction in embryo survival. However, no significant difference ($p > 0.05$) was found in embryo survival between rapid cooling and slow cooling (1°C/min) following the 1 min exposure to -5°C. The results indicated that chilling injury in 64-embryos was more likely to be caused by the period of exposure rather than the act of rapid cooling. It was also demonstrated that the chilling injury was manifest faster at -5°C than at 0°C.

4.2.1.2 Effect of developmental stage, exposure time and temperature on the chilling injury

Fig. 4.3 shows the effect of cooling rate on the chilling sensitivity of zebrafish embryos at 50%-epiboly, 6-somite and prim-6 embryos at 0°C. After 1 h chilling at 0°C, no significant difference ($p > 0.05$) was found in the survival of embryos at both 50%-epiboly and 6-somite stages with the three different cooling rates, whereas, with prim-6 embryos, the effect of cooling rate on embryo survival was highly significant ($p < 0.01$). The reduction of embryo survival was particularly manifested ($p < 0.01$) following rapid cooling. However, when the chilling period at 0°C was as brief as 1 min, rapid cooling did not result in a significant reduction ($p > 0.05$) in embryo survival.

When the embryos were cooled to -5°C, a 1 h exposure resulted in a significant ($p < 0.01$) reduction in embryo survival at all stages when compared with non-chilled control embryos no matter what cooling rate was used (Fig. 4.4), indicating that indirect chilling injury, independent of the cooling rate, was experienced in these embryos. Rapid cooling followed by chilling for 1 h at -5°C resulted in significantly lower ($p < 0.01$ or $p < 0.05$) embryo survival than observed for other two cooling rates for all stages. When the period of exposure to -5°C was reduced to 1 min, there was no significant difference ($p > 0.05$) between the three cooling rates in survival of embryos at any of the three stages (Fig. 4.5). The results suggest that the cold shock injury resulting from rapid cooling in embryos at all three stages, developed only after an extended exposure period (1 h) at -5°C. However cold shock injury was only observed in prim-6 embryos following 1 h exposure at 0°C.

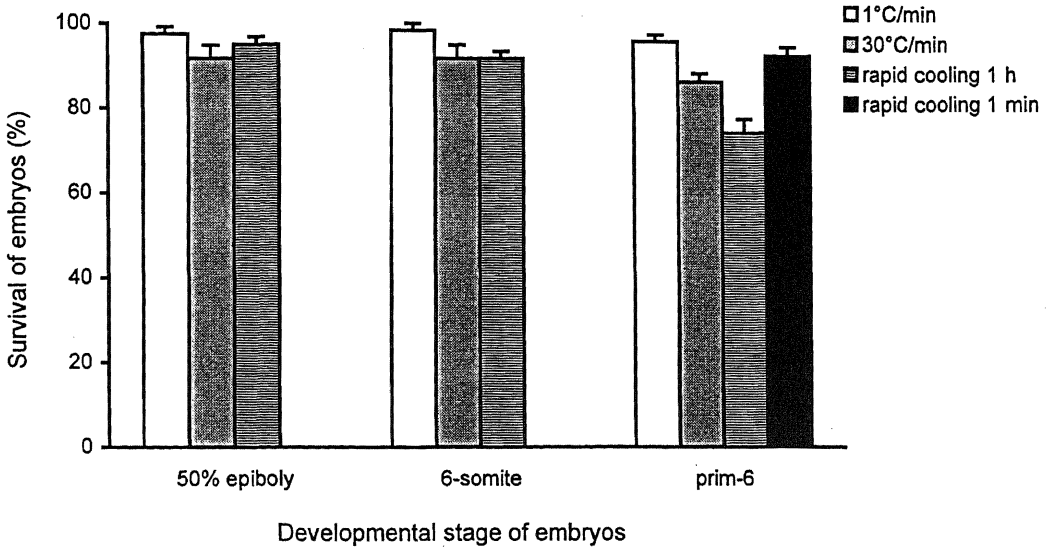


Fig. 4.3 Comparison of effect of cooling rate on the chilling sensitivity of zebrafish embryos at different stages. Embryos were chilled for 1 h or 1 min (only for prim-6 rapid cooling) at 0°C. The survival of non-chilled control was 97.0 ± 1.9%, 96.7 ± 1.1%, and 97.5 ± 1.7% for 50%-epiboly, 6-somite and prim-6 embryos, respectively. The differences were not significant ($p > 0.05$). Values are means ± SEM (n = 6).

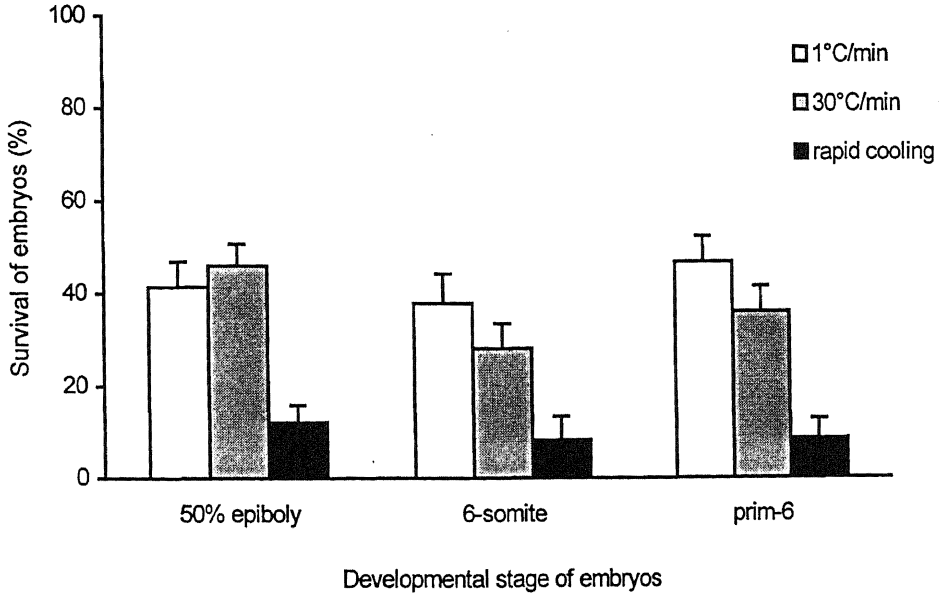


Fig. 4.4 Comparison of effect of cooling rate on the chilling sensitivity of zebrafish embryos at three different stages. Embryos were chilled for 1 h at -5°C. The survival of non-chilled control was 97.0 ± 1.9%, 96.7 ± 1.1%, and 97.5 ± 1.7% for 50%-epiboly, 6-somite and prim-6 embryos, respectively. Values are means ± SEM (n = 6).

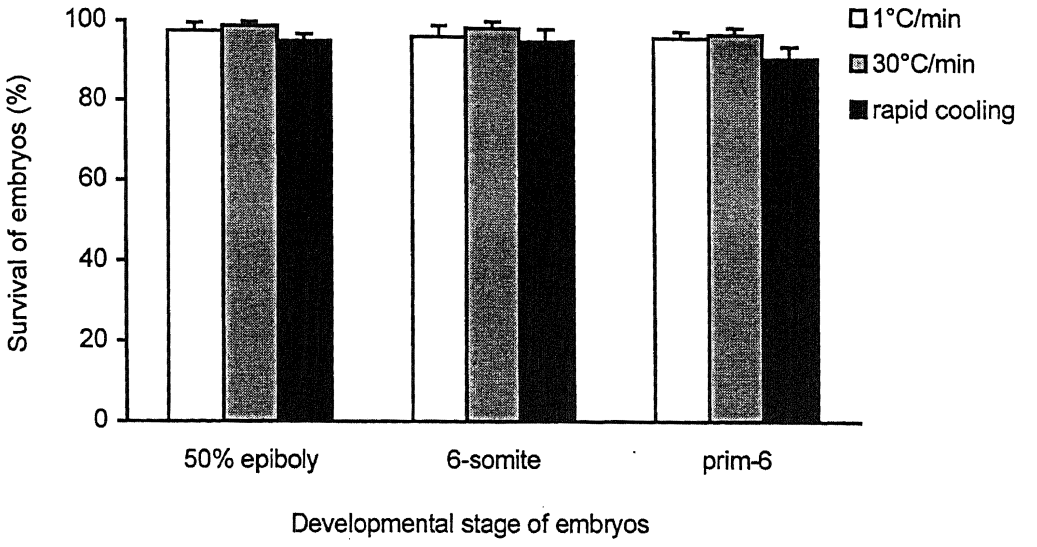


Fig. 4.5 Comparison of effect of cooling rate on the chilling sensitivity of zebrafish embryos at three different stages. Embryos were chilled for 1 min at -5°C. The survival of non-chilled control was 97.0 ± 1.9%, 96.7 ± 1.1%, and 97.5 ± 1.7% for 50%-epiboly, 6-somite and prim-6 embryos, respectively. Values are means ± SEM (n = 6).

4.2.2 Effect of anoxia on the development and survival of zebrafish embryos

Morphological assessment showed that both 50%-epiboly and prim-6 embryos underwent developmental arrest almost immediately after 15 min oxygen deprivation. However, 64-cell embryos did not undergo arrest until they reached sphere stage, indicating that approximately 2 h was required for the onset of developmental arrest. The arrested embryos at sphere stage rather than 64-cell stage were therefore used for the following chilling sensitivity tests. On return to normally oxygenated EM at 26°C, the arrested embryos which remained viable following 4 or 24 h anoxia resumed their normal development.

After 4 h in anoxia, the survivals of the embryos at sphere, 50%-epiboly and prim-6 stages were 95.2 ± 2.8%, 88.3 ± 3.1% and 89.2 ± 4.9%, respectively. There was no significant difference ($p > 0.05$, $n = 6$) in embryo survival when compared with that of their respective aerobic control, which were 96.7 ± 1.5% (sphere), 89.5 ± 4.5% (50%-epiboly) and 95.0 ± 3.4% (prim-6). However, few embryos survived 24 h exposure to anoxia, and

embryo survivals were 6.7 ± 6.1%, 1.7 ± 1.7% and 11.7 ± 6.5% for sphere, 50%-epiboly and prim-6 stage respectively.

4.2.3 Effect of developmental arrest on indirect chilling injury in zebrafish embryos

Fig. 4.6 compares the survival of sphere embryos, which were oxygen-deprived at 64-cell stage, with that of fully aerated embryos as a function of time at 0°C. Although the overall effect of anoxia on embryo survival appeared to be significant ($p < 0.01$), no significant difference ($p > 0.05$) was found in survival between anoxic and aerobic embryos after 20 min chilling at 0°C, and both anoxic and aerobic embryos succumbed to 30 min chilling. The results indicated that anoxia did not increase the ability of the sphere stage embryos to tolerate chilling for a longer period.

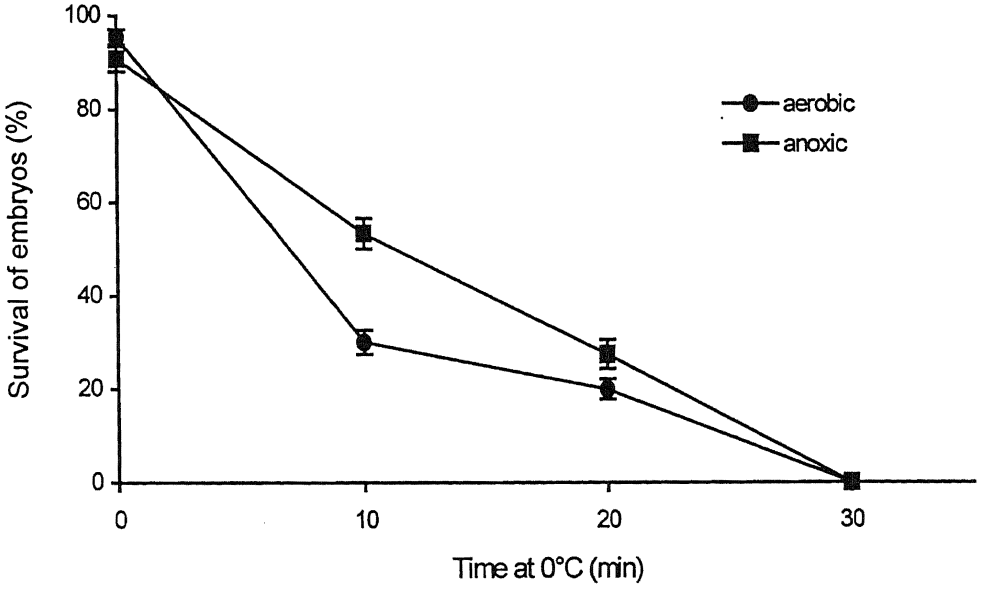


Fig. 4.6 Effect of developmental arrest by anoxia on the indirect chilling injury in sphere stage embryos of zebrafish. Embryos were oxygen-deprived at 64-cell stage and the development was halted at sphere stage following 2 h in anoxia. The development-arrested sphere embryos were then cooled to 0°C for 10, 20 and 30 min before warming and returning to normally oxygenated EM at 26°C. The cooling rate was1°C/min. Values are means ± SEM (n = 6).

Fig. 4.7 shows the comparison of the survival of anoxic and aerobic embryos at 50%-epiboly and prim-6 stages after 1 h exposure to -5°C. Statistically, no difference ($p > 0.05$) was found between the survival of anoxic embryos and that of aerobic embryos at either stage. Thus, anoxia appears to have no effect on the chilling sensitivity of zebrafish embryos at these two stages.

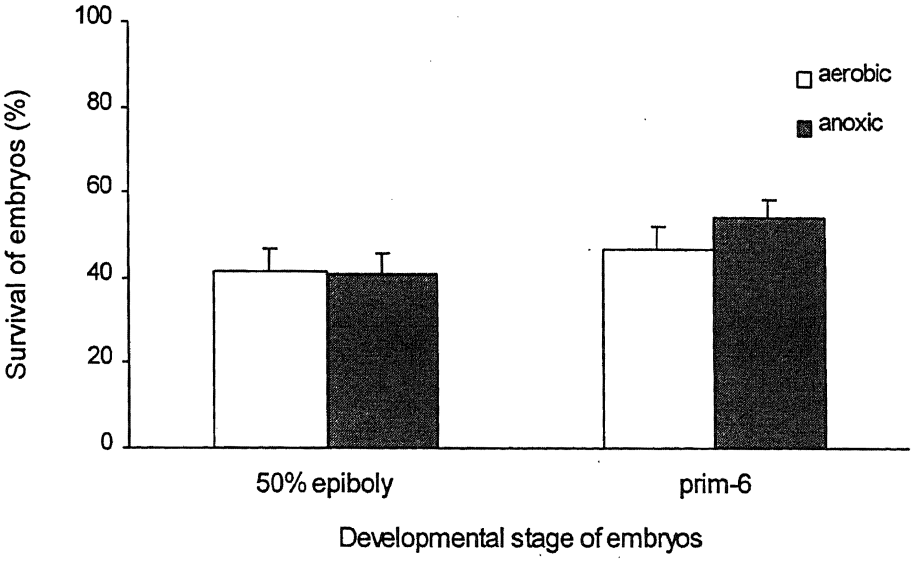


Fig. 4.7 Effect of developmental arrest on the indirect chilling injury in zebrafish embryos at 50%-epiboly and prim-6 stages. Embryos were oxygen-deprived and development-halted at 50%-epiboly or prim-6 stage. They were then cooled to -5°C for 1 h before warming and returning to normally oxygenated EM at 26°C. The cooling rate was 1°C/min. Values are means ± SEM (n = 6).

4.3 Discussion

4.3.1 Cold shock injury in zebrafish embryos

Compared with rapid cooling, slow cooling to 0°C resulted in significantly lower survival of embryos at 64-cell stage, indicating that the 64-cell embryos are likely to be more sensitive to indirect chilling injury than cold shock arising from the rapid cooling (if it occurs in the embryos) because the slow-cooled embryos were exposed to low temperatures for a longer period than those embryos which were cooled rapidly. Short term exposure for 10 sec to -5°C following rapid cooling did not cause a reduction in embryo survival,

suggesting that the chilling injury in 64-cell embryos is unlikely to be a consequence of the act of rapid cooling *per se*. However, following 1 min chilling at -5°C after rapid cooling, the chilling injury was manifested. Here a distinction can not satisfactorily be made between cold shock injury and indirect chilling injury because the latter developed rapidly at -5°C. These findings are similar to those of chilling injury in porcine embryos at morula stage (Pollard and Lerbo, 1994) and bovine oocytes at germinal vesical stage (Martino *et al.*, 1996).

With later stage embryos, following 1 h chilling at 0°C, no chilling injury was observed in either 50%-epiboly or 6-somite embryos after cooling at three different rates. However, chilling injury occurred to prim-6 embryos after 1 h exposure to 0°C and it was associated with the rate of cooling. The injury was particularly manifested following rapid cooling, and cold shock is therefore more likely to be the cause of the injury since slow cooling of 1°C/min did not result in the reduction of survival of prim-6 embryos. Moreover, the cold shock injury was also related to the exposure time and developed only after extended exposure periods (such as 1 h) at 0°C. After cooling to -5°C, the cold shock injury in all three stage embryos was similar to that observed in prim-6 embryos at 0°C. These results demonstrate that zebrafish embryos at 50%-epiboly, 6-somite and prim-6 stages are all susceptible to cold shock injury if rapidly cooled to 0°C or -5°C and held at these temperatures for 1 h. These findings are in agreement with one of the features of cold shock that all cell-types may be considered to be sensitive to cold shock, provided that they are cooled rapidly enough to a sufficiently low temperature (Morris, 1987).

Cold shock injury has been recognised in a wide range of cell-type and tissues including mammalian spermatozoa, eggs, embryos, blood cells and micro-organisms (Morris and Watson, 1984). It appears to be a general phenomenon, differences between cell-types being quantitative (in the rate of cooling and temperature range at which injury is sustained) rather than qualitative (Watson and Morris, 1987). The thermotropic behavior of membrane lipids has been considered to determine the cold shock injury (Morris, 1987), and a number of studies (Holt and Noth, 1988; Drobnis *et al.*, 1993; Arav *et al.*, 1996; Crowe *et al.*, 1999) have supported the theory that lipid phase transition in cell membranes are responsible for cold shock injury. According to this interpretation, rapid rates of cooling cause membrane phase separation which results in more defects than slowly chilled membranes. Hence

rapidly chilled samples would be more likely to produce leaky membranes and cause severe damage to cells.

Another mechanism of cold shock advanced by McGrath (1984) is the thermoelastic stress theory. Because reduced temperature will result in membranes attempting to thermally contract laterally around an essentially incompressible aqueous interior, such membrane shrinkage in cells will result in stress and damage to the membrane. This stress or tension is dependent on the water permeability of the cell and the cooling rate (within the range of 10 to 500°C/min). Faster cooling rate will produce increased tension for a given temperature drop. Therefore cold shock injury is observed only in the case of rapid cooling.

4.3.2 Developmental arrest by anoxia and survival of anoxic embryos

Reduction of the concentration of dissolved oxygen usually causes the retardation of development of fish embryos (Kinne and Kinne, 1962; Garside, 1966). In the present study, anoxia was shown to be able to produce a rapid, but reversible developmental arrest of zebrafish embryos at 50%-epiboly and prim-6 stage, but 2 h is needed to halt the development of 64-cell embryos. Embryos at all three stages can be maintained in anoxia for at least 4 h without a major loss of viability, but the majority of these embryos can not survive 24 h anoxia. These results are on a parallel with the findings of anoxia with *Drosophilia* embryos (Foe and Alberts, 1985) except that *Drosophilia* embryos have significantly different sensitivities to the oxygen deprivation between different stages and older embryos can be kept in an arrested state for a much longer period (such as 36 h). One possible explanation suggested for the effect of anoxia could be that oxygen deprivation is expected to bring about the cessation of oxidative phosphorylation, which results in a large number of different biochemical changes in cells, including a rapid increase in nicotinamide adenine dinucleotide (NADH), ADP, AMP, and inorganic phosphate levels, a rapid decrease in the concentration of ATP, and a major increase in the lactic acid concentration and a concomitant lowering of the intracellular pH (Foe and Alberts, 1985). Later studies showed that the acute acidification of intracellular pH by over 1.0 unit during the transition into anoxia contributes to the depression of biosynthesis in *Artemia franciscana* embryos (Anchorodoguy *et al.*, 1993; Warner *et al.*, 1997). Another mechanism forwarded by Hand (1998) is that direct sensing of molecular oxygen (or oxygen by-products) together with acidification of intracellular pH may be responsible for the down-regulation of biosynthesis

in the mitochondrial compartment of *Artemia franciscana* embryos during the anoxia-induced reversible quiescence state.

4.3.3 Effect of developmental arrest on the indirect chilling injury of zebrafish embryos

Embryos at sphere stage appeared to be somewhat less sensitive to chilling for 10 min exposure at 0°C following the arrest of embryo development, but the reduction of the indirect chilling injury in sphere stage embryos was not significant after an extended chilling period (20 and 30 min) at 0°C. Developmental arrest did not have any significant effect on the chilling sensitivity of embryos at both 50%-epiboly and prim-6 stages at the tested temperature (-5°C) and exposure period (1 h). These findings are in agreement with the results of Mazur *et al.* (1992) with *Drosophila* embryos.

Based on one characteristic of the indirect chilling injury in *Drosophila* embryos, namely that it is preceded by a high-survival shoulder or plateau, Mazur *et al.* (1992) proposed that during the time represented by the shoulder followed by rapid decline, faulty enzymatic products accumulate as concatenated biochemical reactions lose their synchrony at low temperature because different reactions possess different activation energies. If so, the chilling sensitivity of anoxic embryos of which development was reversibly arrested prior to chilling should have been reduced since the concatenated biochemical reactions were supposed to be halted during the development-arrested state. However, the effect of anoxia on chilling sensitivity of 6-h *Drosophila* embryos was quite marginal at subzero temperatures, and it had no beneficial effect on 15-h embryos (Mazur *et al.*, 1992). The indirect chilling injury in zebrafish embryos has been shown to have similar characteristics with a high-survival shoulder followed by rapid decline (Zhang and Rawson, 1995), but again no reduction of chilling sensitivity has been observed in the development-arrested zebrafish embryos during the exposure period at the temperatures tested. Although further study of the effect developmental arrest on indirect chilling injury might be warranted, these preliminary results did not support the hypothesis that the high chilling sensitivity of these embryos resulted from the loss of synchrony of coupled reactions involved in embryological development.

Several other theories have been proposed for the mechanism of indirect chilling injury, but they differ by species and cell type. At low temperatures, microtubules are depolymerised (Behnke and Forer, 1967; Weber *et al.*, 1975; Magistrini and Szollosi, 1980), and cellular processes like cell division in oocytes can be irreversibly disrupted (Magistrini and Szollosi, 1980; Martino *et al.*, 1996); protein can be denatured due to the destabilisation of hydrophobic bonds (Brandts, 1964); and the plasma membrane can suffer lateral phase separation (Morris and Clarke, 1987). However, because a reduction in temperature will simultaneously modify all aspects of cell biology, it is therefore perhaps an oversimplification to expect single determinants to explain the effect of low temperatures on integrated cellular processes, and obviously, in multicellular systems like embryos such processes are more complex. It is interesting to note that after delipidisation early porcine embryos gained tolerance to cooling (Nagashima *et al.*, 1994) and survived cryopreservation in LN_2 (Nagashima *et al.*, 1995), which seems to confirm that the high chilling sensitivity of early porcine embryos is associated with their high intracellular lipid content (Toner *et al.*, 1986; Nagashima *et al.*, 1994). There are abundant lipids in the yolk of fish embryos, and to test whether the high chilling sensitivity of zebrafish embryos could also be associated with their high lipid content, the effect of partial removal of yolk on the chilling injury in zebrafish embryos needs to be determined (see Chapter 5).

4.4 Summary

The effects of cooling rate and developmental arrest on the chilling injury in intact zebrafish embryos were investigated. At 0°C, 64-cell stage embryos had higher survival following rapid cooling than when they were cooled at a slower rate. At -5°C, after just 1 min exposure, the survival of 64-cell embryos decreased greatly following both rapid cooling and slow cooling whilst rapid cooling to -5°C for 10 sec did not cause the reduction of embryo survival, suggesting embryos were more sensitive to indirect chilling injury than cold shock arising from rapid cooling. The effect of cooling rate on the survival of 50%-epiboly and 6-somite embryos was not significant following 1 h exposure at 0°C, but it had a significant effect on survival of embryos at prim-6 stage. However, rapid cooling to 0°C for 1 min did not result in the reduction of embryo survival. Following 1 h chilling at -5°C, rapidly-cooled embryos at 50%-epiboly, 6-somite or prim-6 stages all showed significantly lower survival when compared with their corresponding embryos which were cooled by two slower cooling rates although no reduction of survival was found following 1 min chilling

for all these embryos cooled at the three different rates, This indicated that cold shock injury in prim-6 embryos did not occur instantaneously and was only manifest after an extended period of chilling.

Oxygen deprivation induced developmental arrest almost immediately in both 50%-epiboly and prim-6 embryos, but it did not halt the development of 64-cell embryos until they reached sphere stage. No reduction of embryo survival was observed at any stage following 4 h anoxia although most embryos succumbed to 24 h developmental arrest by anoxia. On return to normally oxygenated EM, surviving embryos resumed their development following the arrest. Anoxia improves the survival of sphere stage embryos following 10 min chilling at 0°C, but the effect was marginal after an extended exposure period at 0°C. The reversible arrest of development did not show any significant effect on the chilling sensitivity of embryos at both 50%-epiboly and prim-6 stages at -5°C.

The above results demonstrate that zebrafish embryos at 50%-epiboly, 6-somite and prim-6 embryos are all sensitive to cold shock injury when they are cooled rapidly to -5°C and exposure for a sufficiently long period (1 h). It is difficult to distinguish cold shock injury from the indirect chilling injury at 0 or -5°C in early stage (64-cell) embryos because of the rapid development of chilling injury in the embryos. The developmental arrest experiments with anoxia did not support the hypothesis that the high chilling sensitivity of zebrafish embryos is associated with high embryo development rate.

CHAPTER 5 EFFECT OF PARTIAL REMOVAL OF YOLK ON THE CHILLING INJURY AND CRYOPROTECTANT TOXICITY IN ZEBRAFISH EMBRYOS

5.1 Introduction

As discussed in Chapter one, factors which are suspected of complicating teleost embryo cryopreservation include (a) their large overall size and resultant low surface-to-volume ratio, retarding water and cryoprotectant efflux/influx; (b) the two compartment nature of the embryo, blastoderm and yolk, and the different osmotic properties of each stucture making it difficult to determine an optimum protocol suitable for both; (c) high chilling sensitivity, precluding conventional controlled slow cooling procedures (Zhang and Rawson, 1995); and (d) permeability barriers to cryoprotectants (Hagedorn *et al.* 1996; Zhang and Rawson, 1996a), resulting in insufficient cryoprotectant permeation into embryos and unsuccessful vitrification (Zhang and Rawson, 1996a; Chapter 3). Harvey (1983) reported that after cooling of intact embryos treated with 2.8 M glycerol to -196°C, most cells of blastoderm appeared intact, but the yolk was misshapen. Hagedorn *et al.* (1996) found that the blastoderm of zebrafish embryos was permeable to cryoprotectant such as DMSO and PG, and the yolk syncytial layer was identified as the barrier to the movement of these cryoprotectants to the yolk. They also characterised a permeability barrier in the yolk compartment of zebrafish embryos (Hagedorn *et al.*, 1998). These findings suggest that factors which complicate the cryopreservation of these embryos are mainly yolk related. Recently, Olive and Wang (1997) reported that to achieve successful cryopreservation of *Nereis virens* larvae, the optimum developmental stage is that at which the embryonic yolk is almost digested but the larvae have not started to feed. The high yolk mass appears to be responsible for the difficulty in yolk-laden embryo cryopreservation. Moreover, several workers have suggested that the sensitivity of porcine embryos to lowered temperatures relates to their relatively high lipid content (Toner *et al.*, 1986; Mohr *et al.*, 1981). After removal of lipids, porcine embryos became more tolerant to chilling (Nagashima *et al.*, 1994) and survived cryopreservation in liquid nitrogen (Nagashima *et al.*, 1995).

In fish embryos, lipids are the second most abundant dry constituent and are mainly located in the yolk (Heming and Buddington, 1988). In order to determine if the high chilling sensitivity of zebrafish embryos could also be associated with their high lipid content, yolk contents were partially removed and the chilling sensitivity of the partially yolk-reduced embryos was compared with dechorionated control embryos. The cold shock injury arising from rapid cooling was also studied in yolk-reduced embryos. Yolk removal was presumed to be most detrimental to early stage fish embryos, and experiments were therefore carried out to identify the appropriate embryo developmental stages which could tolerate the damage arising from the yolk removal. A pilot experiment showed that zebrafish embryos during/before gastrula period were very susceptible to physical damage, so yolk removal experiments were carried out only with embryos during/after segmentation period. Five embryo developmental stages (6-somite, 26-somite, prim-6, prim-15 and high-pec) were examined.

If embryos could survive partial removal of their yolk, consideration would also be given to exploring the possibility of introducing cryoprotectants into the yolk during the yolk removal procedures, and the next question would be whether or not the yolk-reduced embryos became less tolerant to cryoprotectants. Methanol and PG were reported to be less toxic to zebrafish embryos than other cryoprotectants (Zhang and Rawson, 1996), and DMSO is the most commonly used cryoprotectants. Previous studies on the toxicity of cryoprotectants (Zhang and Rawson, 1996) also found that the maximum no observed effect concentration of methanol, PG and DMSO to intact prim-6 embryos was 2, 3 and 2 M respectively at 22°C after 30 min exposure. The intended introduction of cryoprotectants into embryos by puncturing the yolk sac may result in increased cryoprotectant penetration, therefore it is possible that the toxicity of cryoprotectants to the embryos could be increased by using this procedure. The toxicity of methanol, PG and DMSO at a concentration of 1 or 2 M was examined in these experiments. Appropriate stages were chosen to test the toxicity of the cryoprotectants based on the results of embryo survival following multi-punctures of the yolk.

5.2 Results

5.2.1 Effect of partial removal of yolk on embryo survival

After partial removal of yolk, the survival of yolk-reduced embryos at all developmental stages was significantly reduced ($p < 0.01$) when compared with that of their corresponding control (dechorionated) embryos (Fig. 5.1). However, the effect of developmental stages on the survival of yolk-reduced embryos was highly significant ($p < 0.01$). Whilst all embryos at 6-somite stage died and only 7.9% 26-somite embryos survived, 81.3% embryos survived at high-pec stage. Later embryo developmental stages were better able to survive partial yolk removal. Another experiment with 6-somite and prim-6 stage embryos showed that tiny punctures (without considerable yolk loss) did not cause significant reduction ($p > 0.05$) of survival of prim-6 embryos, but it resulted in a significant lower ($p < 0.01$) survival (45.0 ± 10.0%) of 6-somite embryos. The results indicated that younger embryos such as 6-somite stage were highly sensitive to the physical damage arising from the multi-punctures, whilst older embryos such as prim-6 stage were not affected by this physical damage. As yolk-

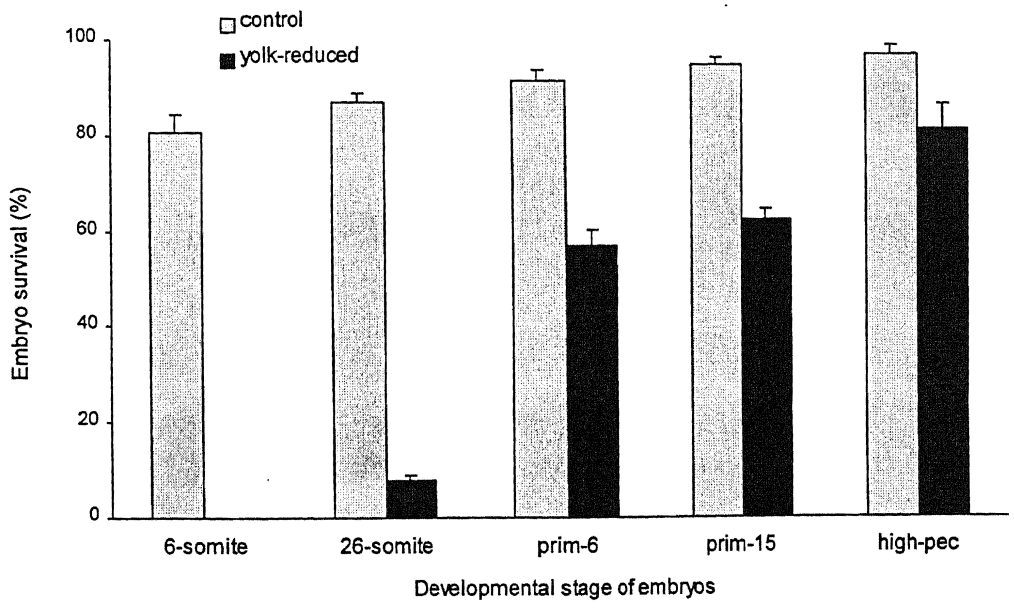
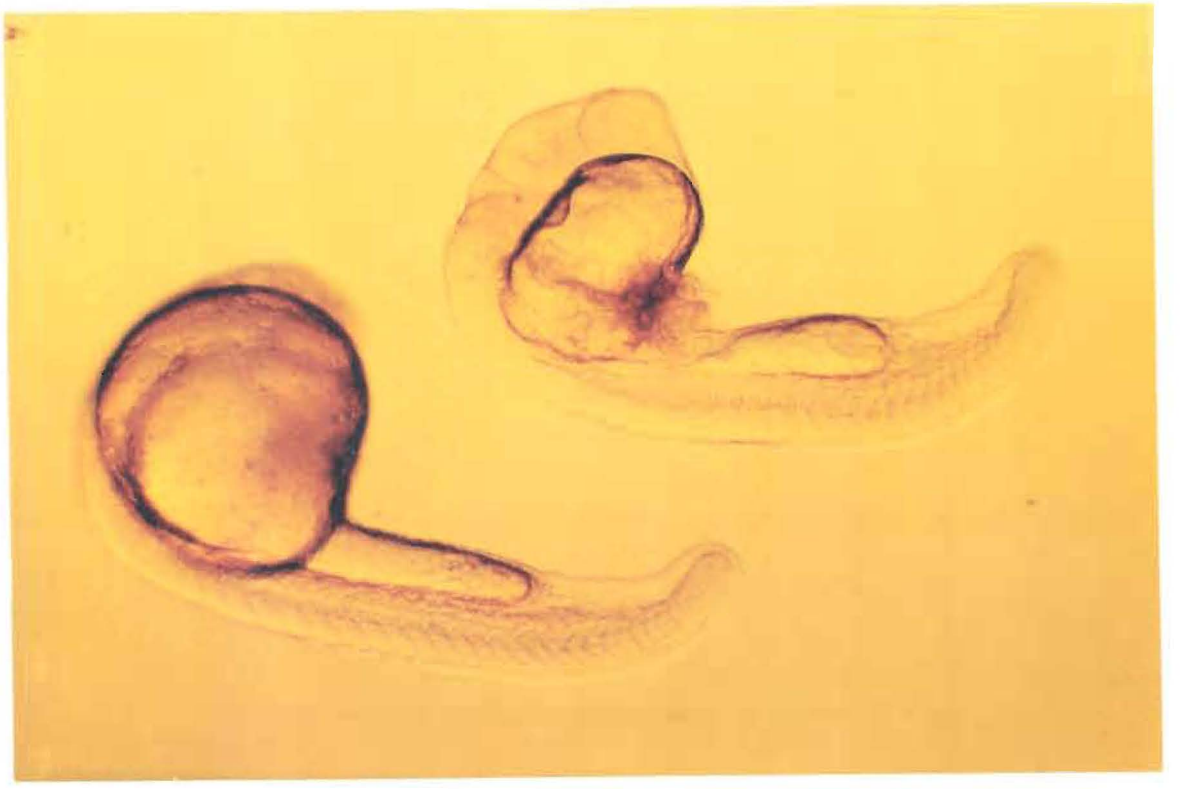


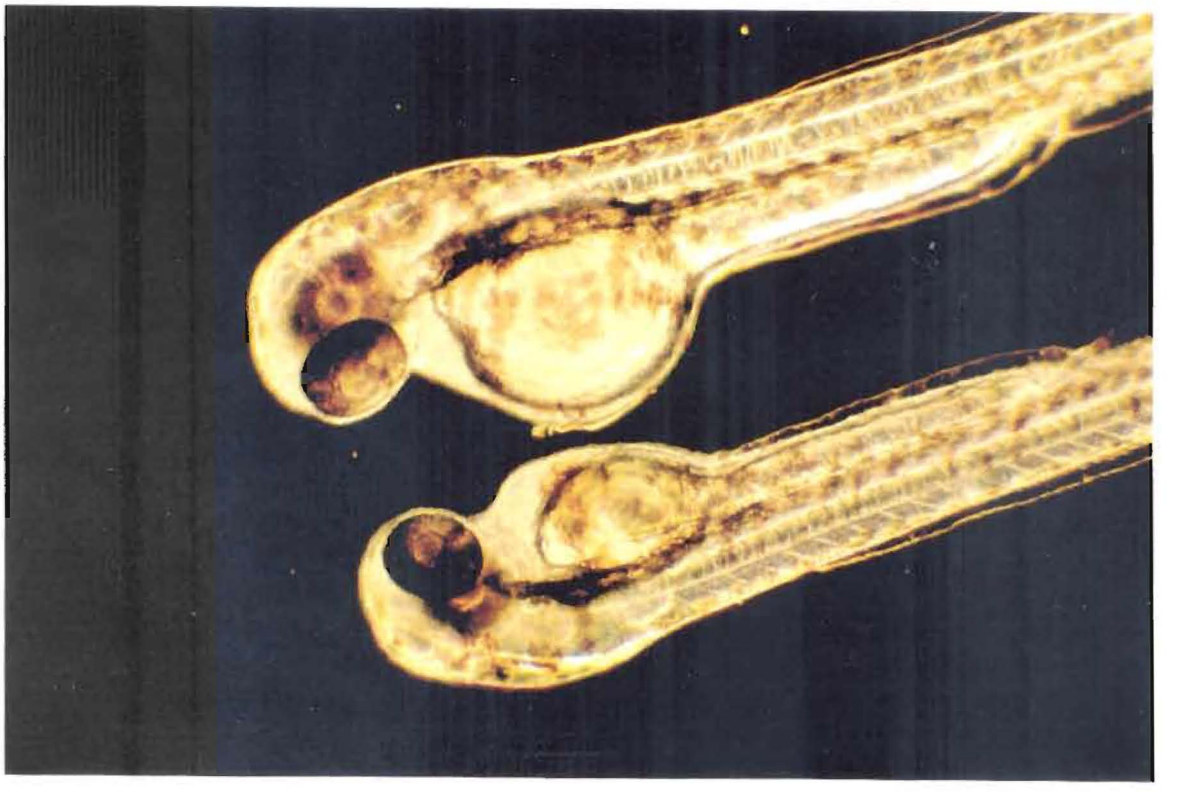
Fig. 5.1 Effect of partial removal of yolk on the survival of embryos at five developmental stages. Approximately 50 to 75% of yolk content was removed. Embryo survival is defined as the percentage of embryos with developmentally normal appearance after 3 days of maintenance in EM at 26°C. The survival of yolk-reduced embryos at 6-somite stage was zero. Values are means ± SEM (n = 6).



A: yolk-reduced and dechorionated embryos at 26-somite stage

B: yolk-reduced and dechorionated embryos at prim-6 stage

C: yolk-reduced and dechorionated embryos at prim-15 stage



D: yolk-reduced embryos and dechorionated at high-pec stage

Fig. 5.2 Comparison of yolk-reduced embryos and dechorionated (control) embryos at four developmental stages. A: 26-somite stage; B: prim-6 stage; C: prim-15 stage; D: High-pec stage. Photos were taken at about half an hour following partial removal of yolk except high-pec stage at 24 h culture at 26 ± 1 °C. × 60.

reduced high-pec embryos have the highest survival and prim-6 was the earliest stage to have better than 50% survival following the partial removal of yolk, these two embryo stages were chosen to determine the effect of partial removal of yolk on their sensitivity to chilling. Yolk-reduced prim-6 and punctured 6-somite embryos were used for the following cryoprotectant toxicity test since nearly half of 6-somite embryos survived the multi-punctures without yolk loss and also at this stage embryos have not developed an epidermis (Westerfield, 1995), which might be another permeability barrier to water and cryoprotectants at later stages. Fig. 5.2 shows the comparison between yolk-reduced embryos and that of dechorionated control embryos at several developmental stages.

5.2.2 Effect of culture time on the chilling sensitivity of yolk-reduced embryos

Chilling sensitivity of dechorionated and yolk-reduced embryos at high-pec stage after different culture periods following removal of yolk is shown in Fig. 5.3. The effect of

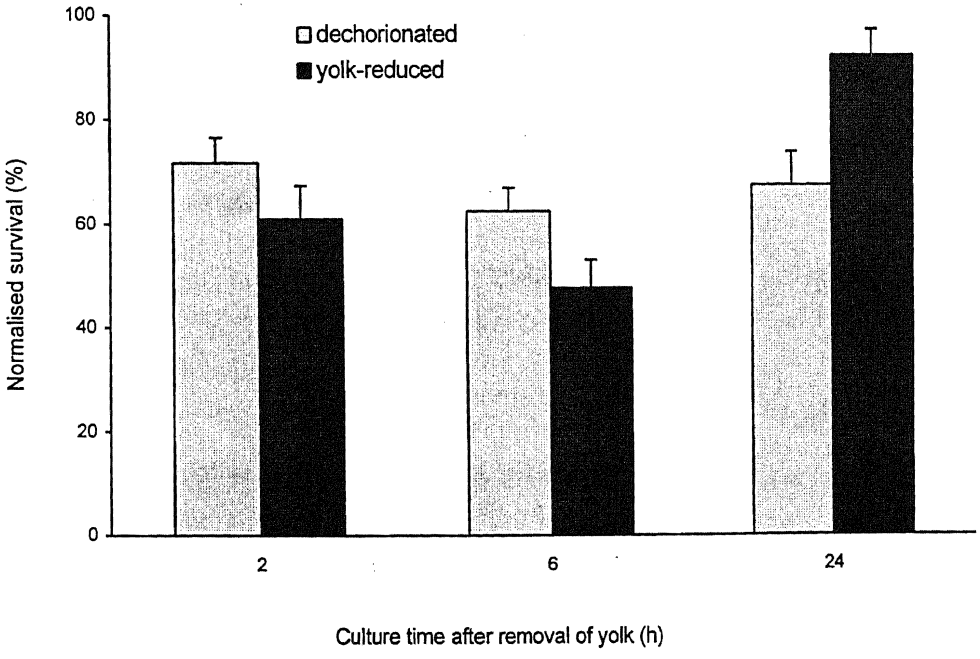


Fig. 5.3 Effect of culture time on the chilling sensitivity of yolk-reduced embryos of zebrafish. High-pec stage embryos were yolk-reduced and cultured in EM at 26 ± 1°C for 2, 6 or 24 h before being chilled at 0°C for 6 h. Embryo survival was normalised with respect to non-chilled controls, which averaged 97.7 ± 1.8% (dechorionated control) and 81.6 ± 5.3% (yolk-reduced control) respectively. Values are means ± SEM (n = 6).

culture time on the chilling sensitivity of yolk-reduced embryos are highly significant ($p < 0.01$). Following 2 or 6 h culture periods, no significant differences ($p > 0.05$) were seen in normalised survival between dechorionated and yolk-reduced groups, although the average percentage survivals of the yolk-reduced embryos were lower than that of the controls. However, after 24 h culture, the normalised survival of yolk-reduced embryos increased significantly ($p < 0.05$) to 92.3 ± 11.9% when compared with dechorionated embryos of 67.5 ± 15.4%. The results indicated that the chilling sensitivity of yolk-reduced embryos was significantly reduced after 24 h culture following removal of yolk.

5.2.3 Effect of the partial removal of yolk on the chilling sensitivity of embryos

The survival of yolk-reduced embryos at prim-6 and high-pec stages after 6 or 10 h chilling at 0°C is shown in Fig. 5.4. After 6 h chilling the normalised survival of both embryo stages was significantly increased ($p < 0.05$) when compared with their

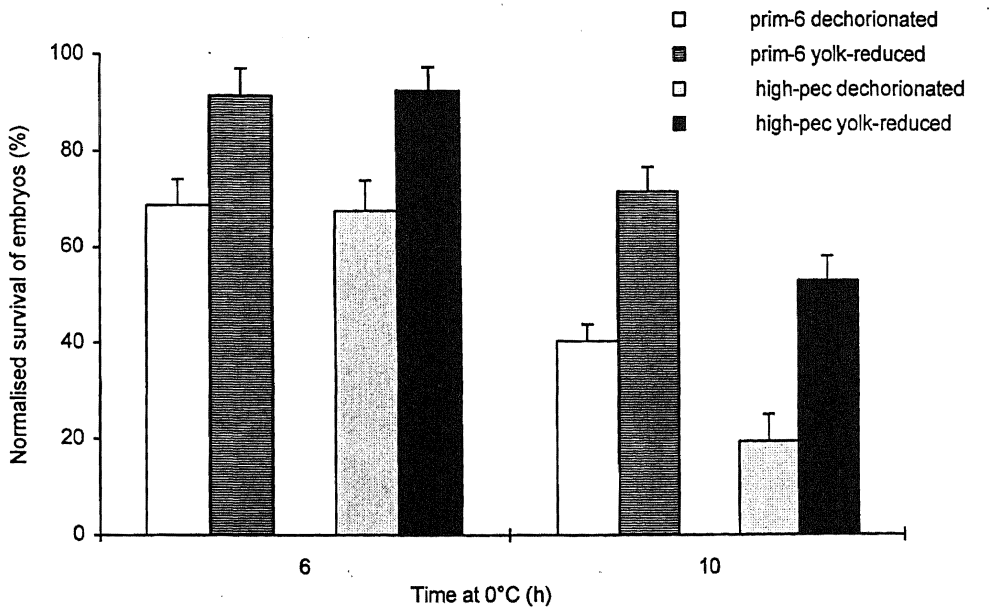


Fig. 5.4 Effect of partial removal of yolk on the chilling sensitivity of zebrafish embryos. Embryos were cultured in EM at 26 ± 1°C for 24 h following the removal of yolk before chilling at 0°C. Embryo survival was normalised with respect to corresponding non-chilled controls, which averaged 99.2 ± 0.8% (prim-6 dechorionated), 80.0 ± 2.6% (prim-6 yolk-reduced), 99.2 ± 0.8% (high-pec normal dechorionated) and 87.5 ± 1.7% (high-pec yolk-reduced) respectively. Values are means ± SEM (n = 6).

dechorionated controls. The significance was greater ($p < 0.01$) when embryos were chilled for 10 h. The results indicate that partial removal of yolk enhanced the tolerance of zebrafish embryos to chilling (at 0°C), and this effect was more pronounced with a longer exposure period.

5.2.4 Effect of the partial removal of yolk on cold shock of zebrafish embryos

Fig. 5.5 compares the normalised survival of yolk-reduced high-pec embryos with dechorionated high-pec embryos after rapid cooling to -5°C as a function of time. After 10 min at -5°C, no obvious reduction of embryo survival was observed in both dechorionated and yolk-reduced embryos. However, when the chilling time was extended to 30 min, the survival of dechorionated embryos was decreased significantly ($p < 0.01$), whilst the survival of yolk-reduced embryos remained similar ($p > 0.05$) to that of their non-chilled controls. After 1 h exposure to -5°C, the survival of both dechorionated and yolk-reduced embryos dropped significantly ($p < 0.01$), but yolk-reduced embryos had higher ($p < 0.05$) normalised survival than dechorionated embryos. The overall effect of partial removal of yolk on the normalised survival of rapidly-cooled embryos was highly significant ($p < 0.01$).

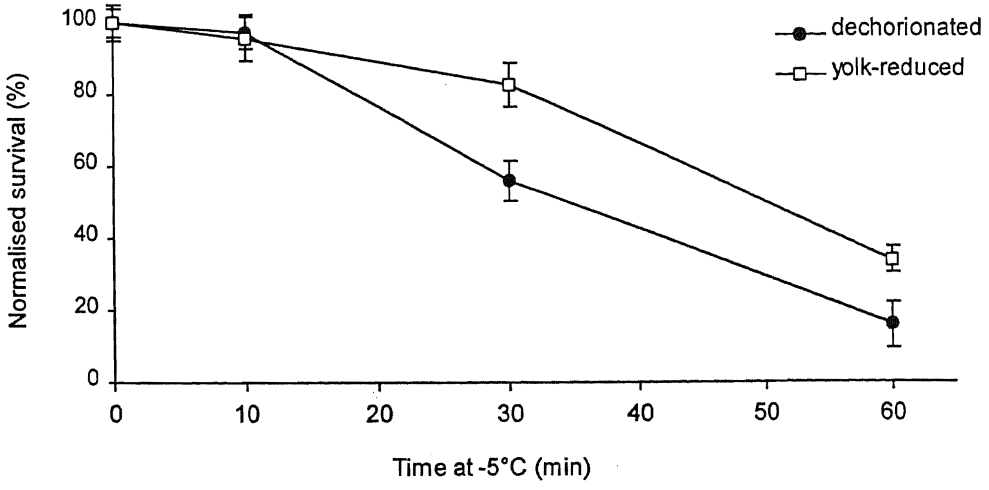


Fig. 5.5 Comparison of the effect of rapid cooling on the normalised embryo survival between dechorionated and yolk-reduced embryos at high-pec stage as a function of time at - 5°C. Embryos were cultured in EM at 26 ± 1°C for 24 h following the removal of yolk at prim-6 stage before chilling at -5°C. Embryo survival was normalised with respect to non-chilled controls, which averaged 94.2 ± 4.9 % and 86.7 ± 4.0% for dechorionated and yolk-reduced embryos, respectively. Values are means ± SEM (n = 6).

When the cooling rate was -1°C/min, 1 h exposure to -5°C did not result in significant reduction of embryo survival in either group, and the normalised survivals were 96.4 ± 4.4 and 98.7 ± 4.1% for dechorionated and yolk-reduced embryos, respectively. These results showed that yolk-reduced embryos appeared to be less sensitive than dechorionated embryos to the chilling injury arising from rapid cooling. This indicates that partial removal of yolk improves the tolerance of zebrafish embryos to cold shock.

5.2.5 Toxicity of cryoprotectants to yolk-reduced prim-6 embryos

5.2.5.1 Effect of cryoprotectants and exposure time

Following treatment with 1 M methanol, DMSO or PG for 30, 60 and 120 min at room temperature, the normalised survival of both yolk-reduced and dechorionated embryos at prim-6 stage is shown in Fig. 5.6. ANOVA reveals that the effect of exposure periods to all

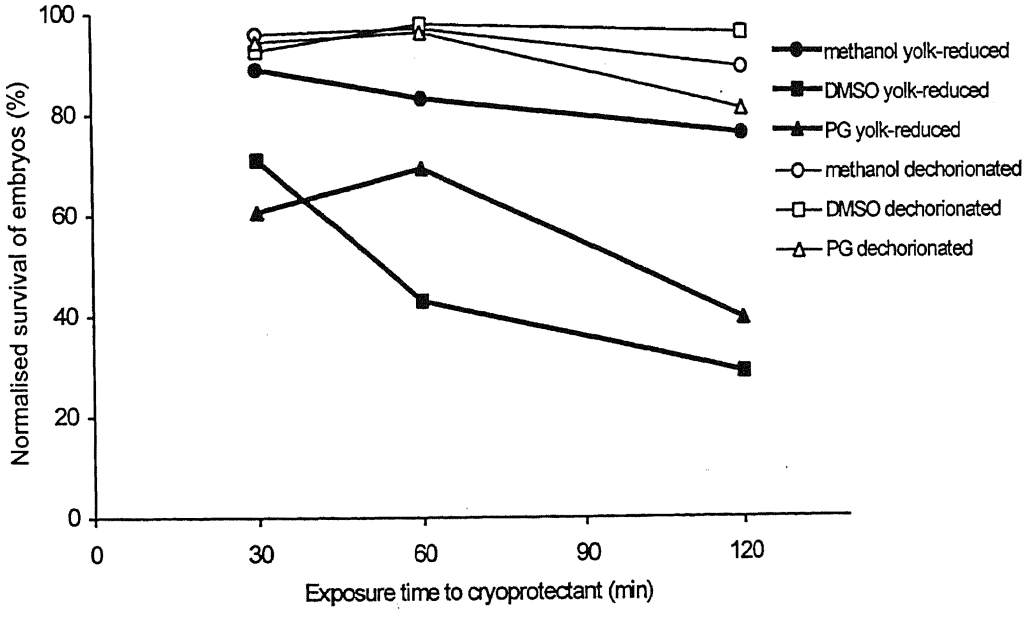


Fig. 5.6 Comparison of cryoprotectant toxicity to dechorionated (control) and yolk-reduced prim-6 embryos. Prim-6 embryos were punctured in 1 M methanol, DMSO or PG solution and more than half of the yolk content was released. The embryos were exposed to 1 M corresponding cryoprotectant solution for 30, 60 and 120 min at room temperature before they were washed with EM. Dechorionated embryos were treated in the same way except that they were not punctured. Embryo survival was normalised with respect to non-treated controls, which averaged 88.0 ± 3.4%and 86.0 ± 7.0% for dechorionated and yolk-reduced embryos, respectively. Values are means ± SEM (n= 5). The error bars of SEM are not shown in the graph. The values of SEM are in the range of 2.6 to 12.1.

the three cryoprotectants on the normalised survival of dechorionated embryos was not significant ($p > 0.05$), indicating that at a concentration of 1 M, none of the three cryoprotectants was toxic to dechorionated prim-6 embryos. However, the survival of yolk-reduced prim-6 embryos was decreased significantly ($p < 0.05$) following 30 min exposure to either 1 M DMSO or 1 M PG. The effect of exposure time on embryo survival was significant and it was more pronounced with 1 M DMSO ($p < 0.01$). Both 1 M DMSO and 1 M PG were deleterious to yolk-reduced prim-6 embryos even with the shortest exposure period (30 min), and their toxicity increased with the exposure time. However, 1 M methanol proved to be non-toxic ($p > 0.05$) to the yolk-reduced embryos over the exposure periods tested.

5.2.5.2 Effect of methanol concentration

The toxicity of methanol at different concentrations (1 to 3 M) and 3 M methanol in combination with 0.5 M sucrose to yolk-reduced prim-6 embryos is given in Fig. 5.7. The effect of the concentration of methanol on the normalised survival of yolk-reduced prim-6

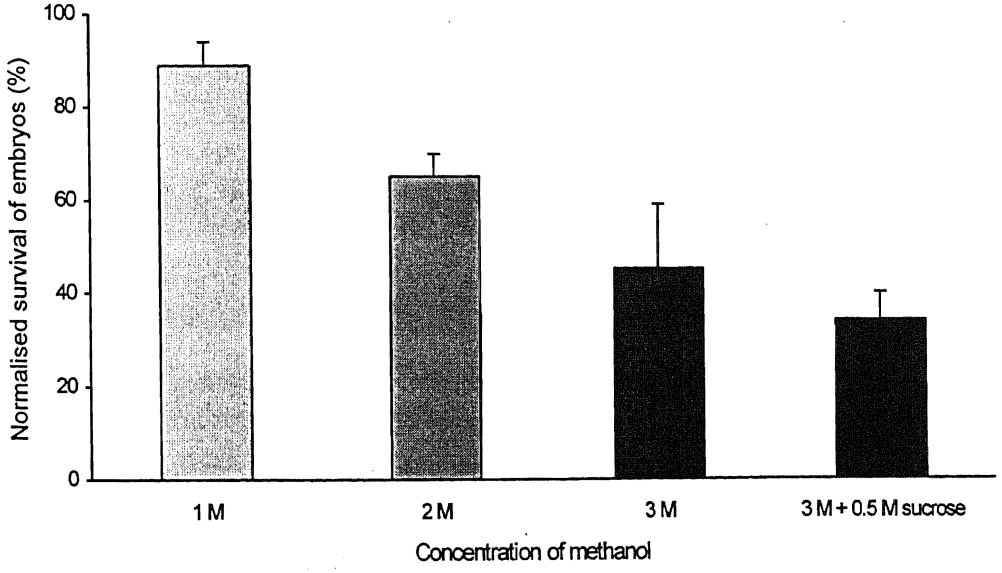


Fig. 5.7. Effect of the concentration of methanol and sucrose on the methanol toxicity to yolk-reduced prim-6 embryos. Prim-6 embryos were punctured in 1 M methanol and more than half of yolk content was released. The embryos were then exposed to 1, 2, or 3 M methanol, or 3 M methanol + 0.5 M sucrose solution for 30 min at room temperature (23 ± 2°C) before they were washed with EM. Embryo survival was normalised with respect to non-treated yolk-reduced embryos, which averaged 73.0 ± 8.9%. Values are means ± SEM (n = 5).

was significant ($p < 0.01$). The normalised survival of the embryos was decreased to 65.1 ± 4.9 and 45.2 ± 13.9 % respectively after 30 min exposure to 2 M and 3 M methanol at room temperature. The addition of 0.5 M sucrose further reduced the embryo survival to 34.2 ± 5.8%, although the difference was not significant ($p > 0.05$) when compared with the value of 3 M methanol treated embryos. The results showed that the toxicity of methanol to yolk-reduced prim-6 embryos increased with concentration and 0.5 M sucrose did not reduce the toxicity of 3 M methanol.

5.2.6 Toxicity of cryoprotectants to punctured 6-somite embryos

As showed in Fig. 5.8, 1 M methanol, PG or DMSO appeared not to be toxic to dechorionated 6-somite embryos, for which normalised survival remained 91.5 ± 4.7, 97.1± 5.7, and 95.8 ± 8.3% respectively after 30 min exposure at room temperature. However, whilst exposure to 1 M PG did not cause a significant ($p > 0.05$) reduction of normalised

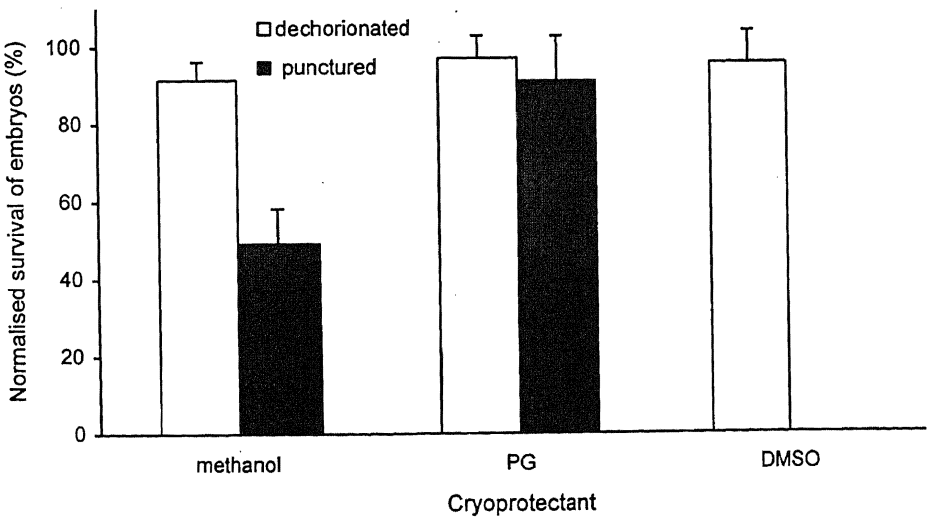


Fig. 5.8 Comparison of cryoprotectant toxicity to dechorionated and punctured 6-somite embryos. Six-somite embryos were cautiously punctured with a sharp micro-needle in 1 M methanol, PG or DMSO solution. Little yolk content was lost from the embryos during multi-punctures. After 30 min exposure to 1 M corresponding cryoprotectants, the punctured embryos were then washed several times with EM. Embryo survival was normalised with respect to non-treated controls, which averaged 77.0 ± 5.8% and 45.0 ± 10.0% for dechorionated and punctured embryos, respectively. There was no survival of punctured embryos following the DMSO treatment. Values are means ± SEM (n= 5).

survival of punctured 6-somite embryos when compared with that of dechorionated embryos, the normalised survival of punctured embryos was reduced to 49.2 ± 9% after 30 min exposure to 1 M methanol, and no punctured embryos survived 30 min exposure to 1 M DMSO. These results demonstrate that at the same concentration of 1 M, the three tested cryoprotectants appeared to have very different ($p < 0.01$) toxicities to punctured 6-somite embryos, whilst none of them were toxic to dechorionated 6-somite embryos. The increased toxicity of methanol or DMSO to punctured embryos may indicate the increased penetration of cryoprotectants into the embryos.

5.3 Discussion

5.3.1 Survival of yolk-reduced embryos

The reduction in the percentage of embryo survival resulting from partial yolk removal may have different causes at different embryo stages. For later stages such as prim-6 embryos, the reduction in embryo survival appeared to be mainly attributable to the removal of yolk *per se*, but in earlier stages like 6-somite, the death of embryos is most likely a consequence of both the loss of yolk content and the mechanical damage arising from the micro-needle puncture of the yolk compartment. Yolk provides soluble nutrients to the cells of the rapidly developing blastoderm and contributes ribosome and membranous material to the embryonic cells for their subsequent differentiation (Thomas, 1968). Removal of yolk during embryo development was presumed to have a significant effect on the normal development of the embryos. It is therefore interesting to find that more than half of post prim-6 stage zebrafish embryos survived after over 50% of the yolk was removed. Results from the present study indicate that the survival of the yolk-reduced embryos is stage dependent, and that there may be a critical developmental stage at which the damage arising from partial removal of yolk could be tolerated and their normal development be unaffected from this stage onwards. Although micro-needle punctures of the yolk compartment caused large membrane disruptions in the embryos, the embryos/cells may be capable of employing a resealing mechanism. Ca^{2+}-dependent exocytotic fusion events were reported to contribute to the resealing of the membrane disruptions (Bi *et al.*, 1995; McNeil and Steinhardt, 1997; Miyake and McNeil, 1995; Terasaki *et al.*, 1997). Further studies need to be carried out on the long term effect of partial removal of yolk on the development of these embryos.

5.3.2 Reduction of chilling injury in yolk-reduced embryos

The present study demonstrates that there is a critical period during which embryos must be cultured under their normal conditions following yolk removal to ensure their recovery. After 2 or 6 h culture, the yolk-reduced embryos at prim-6 and high-pec stages did not show improved tolerance to chilling when compared with their controls, whereas after 24 h culture the sensitivity to chilling of yolk-reduced embryos was significantly reduced. These results suggested that the yolk-reduced embryos fully recovered from their injury after 24 h incubation in EM and their ability to tolerate lowered temperatures was increased.

Chapter 4 has shown that the indirect chilling injury of zebrafish embryos is unlikely to be a consequence of the loss of synchrony of coupled reactions involved in embryological development, and cold shock injury is manifested when they are cooled rapidly to a sufficiently low temperature (e.g. -5°C) and exposure to that temperature for a extended period (1 h). It is demonstrated in this study that both indirect chilling injury and cold shock injury can be reduced by partial removal of yolk. This indicates that both types of chilling injury are related to the yolk and they may have a similar injury mechanism. A number of theories have been proposed to explain the mechanism of both chilling injuries (see Chapter 4). The hypothesis that the phase transition temperature of membrane phospholipids is related to survival after chilling has been confirmed in a range of mammalian spermatozoa (Holt and North, 1984; Parks *et al.,* 1992; Drobnis *et al.,* 1993), bovine oocytes (Arav *et al.*, 1996) and human platelets (Crowe *et al.,*1999). Lateral phase separation of membrane lipid, which occurs when cells are cooled below the phase-transition temperature, is believed to result in a series of damaging processes, such as the formation of 'packing faults' between lipid domains of different phases (Jain, 1983) and the occurrence of nonbilayer lipid (Quinn, 1985), which has serious repercussions with regard to membrane functions and integrity. The thermotropic lipid phase transition is associated with the lipid composition of the membranes (Morris and Clarks, 1987; Quinn, 1992; Drobnis *et al.,* 1993). The lipids, abundant in the yolk of zebrafish embryos, are converted into structural components such as cell membrane or channelled into energy production during embryo development (Heming and Buddington, 1988). Changing the lipid content of zebrafish embryos by removing the yolk may result in changes in the lipid composition of the membranes thereby possibly

modifying the phase-transition properties of membrane lipids and limiting the extent of the phase separation and reducing the damages during chilling.

5.3.3 Cryoprotectant toxicity to yolk-reduced or punctured embryos

The cryoprotectant toxicity study showed that both yolk-reduced prim-6 and punctured 6-somite embryos became less tolerant to cryoprotectants when compared with dechorionated control embryos. Multi-puncturing the yolk sac of embryos probably increased cryoprotectant penetration into the embryos and the toxicity of the cryoprotectant to the yolk-reduced or punctured embryos was therefore manifested at lower concentrations. Methanol was shown to be less toxic to the yolk-reduced embryos than PG and DMSO. These results are different from previous cryoprotectant toxicity studies (Zhang and Rawson, 1996) with *intact* zebrafish embryos which showed that on a molar-equivalency basis PG was less toxic than methanol at room temperature. One reason could be that the degree of PG penetration was much less than methanol for intact embryos (Zhang, 1994), and in yolk-reduced embryos multi-punctures might result in more PG permeation into the embryos than methanol and hence increased toxicity of PG. When the concentration was increased to 2 M, methanol appeared to be toxic to the yolk-reduced embryos. This is in agreement with Zhang's observation (Zhang, 1994) that 2 M methanol exhibited toxic effects on dechorionated heart-beat (prim-6) stage embryos although it was shown to be non-toxic to intact heart-beat (prim-6) stage embryos. Sucrose (0.5 M) was reported to be able to reduce the toxicity of 3 M methanol to intact prim-6 zebrafish (Zhang, 1994) and 1 or 2 M methanol to intact Indian major carp (*Labeo rohita, Catla catla, and Cirrhinus mrigala*) embryos (Ahammad, *et al.*, 1998). However, in the present study, the addition of 0.5 M sucrose did not enhance the tolerance of the yolk-reduced embryos to 3 M methanol. This discrepancy may be attributable to the morphological differences between the intact and the yolk-reduced embryos. In the case of intact fish embryos, sucrose does not penetrate the outer chorion (Zhang and Rawson, 1993a), whereas in yolk-reduced embryos, it is not only in direct contact with the plasma membrane but also could enter the yolk when it was punctured. To better understand the different effect of sucrose in the two cases, further studies need to be carried out to investigate the mechanism for the reduction of toxicity by sucrose when combined with cryoprotectants.

In the case of 6-somite stage embryos, whilst 1 M PG was found not to be toxic to either dechorionated or punctured embryos, punctured embryos became more sensitive to 1 M methanol. The toxicity of DMSO was more pronounced to punctured 6-somite embryos than that of methanol or PG and all punctured embryos succumbed to 30 min exposure to 1 M DMSO although it appeared to be innocuous to dechorionated embryos. These results are consistent with previous findings (Zhang, 1994) that on a molar-equivalency basis DMSO is more deleterious to intact heart-beat embryos than either PG or methanol. However, Hagedorn *et al.* (1997) reported that neither 1.5 M methanol nor DMSO was toxic to dechorionated 3-somite embryos, and 1.5 M PG appeared to be somewhat toxic to the same embryos. When compared with yolk-reduced prim-6 embryos, punctured 6-somite embryos were shown to be less tolerant to 1 M methanol and more tolerant to 1 M PG.

5.3.4 Cryobiological implications

As previously indicated (Zhang and Rawson, 1996a), the main obstacles to the successful cryopreservation of zebrafish embryos are their high sensitivity to chilling and their low permeability to cryoprotectants. The high chilling sensitivity of these embryos increases at temperature below 0° (Zhang and Rawson, 1995) and precludes the use of controlled slow cooling for their cryopreservation. The present study demonstrates that the chilling injury can be reduced after partial removal of their yolk and therefore offers new hope when controlled slow cooling is considered. However, whether or not the chilling sensitivity of zebrafish embryos can be reduced sufficiently after partial removal of the yolk to enable the embryos to tolerate long exposure time to chilling during slow-cooling procedures needs further investigation. It will be necessary to determine the kinetics of chill injury down to the temperatures at which intraembryonic water freezes as determined in *Drosophila* embryos (Mazur, *et al.*, 1992). Moreover, whichever method is employed for fish embryo cryopreservation, the embryos in question have to be sufficiently permeable to water and cryoprotectants. After 24 h culture at 26 ± 1°C, the yolk-reduced prim-6 embryos have developed to high-pec stage, which may constitute a different permeability problem due to the epithelia developed in the embryos. Further studies are therefore needed to investigate cryoprotectant permeability of the yolk-reduced embryos at these stages.

One approach to tackling the low cryoprotectant permeation into the yolk could be to introduce cryoprotectants to the yolk sac by multi-punctures. The present cryoprotectant

toxicity study showed that yolk-reduced prim-6 and punctured 6-somite embryos can tolerate 1 M methanol and PG respectively. Although the chilling sensitivity of these embryos may remain high, vitrification could still be the hope for overcoming the problem if the yolk-reduced or punctured embryos became sufficiently permeable to these cryoprotectants. At the same time, the reduction of yolk size could also benefit the application of vitrification to yolk-reduced embryos. However, further studies of the cryoprotectant penetration in these embryos is needed.

5.4 Summary

The effects of partial removal of yolk on embryo survival, chilling injury and cryoprotectant toxicity in zebrafish embryos were investigated. Dechorionated embryos were punctured with a sharp micro-needle and approximately 50% to 75% of yolk content was released following multiple punctures. The survival of yolk-reduced embryos was stage dependent: no embryos at 6-somite stage survived, only 7.9% of 26-somite embryos survived, whereas 56.7% of prim-6, 62.4% of prim-15, and 81.3% of high-pec embryos survived after partial removal of yolk. After 24 h culture following the removal of yolk, the yolk-reduced embryos at both prim-6 and high-pec stages showed significantly less sensitivity to chilling at 0°C than corresponding controls (dechorionated embryos) and the difference was more pronounced after a longer period (10 h) of chilling. Yolk-reduced high-pec embryos also showed higher embryo survival than dechorionated embryos when they were cooled rapidly to -5°C, suggesting that partial removal of yolk enhanced the tolerance of embryo to cold shock injury arising from rapid cooling. The cryoprotectant toxicity study showed that yolk-reduced high-pec embryos were more sensitive to 1 M PG or DMSO at room temperature than dechorionated embryos. However, 1 M methanol appeared to be non-toxic to the yolk-reduced embryos within the tested exposure periods (30 -120 min). At a concentration of 2 or 3 M, methanol was found to be toxic to the yolk-reduced embryos, and the combination of 0.5 M sucrose did not reduce the toxicity of 3 M methanol. Punctured (without loss of yolk) 6-somite embryos appeared to be much more sensitive to 1 M methanol or DMSO than dechorionated embryos, whilst they were shown to tolerate to 1 M PG treatment for 30 min at room temperature.

The results of this study showed that the high sensitivity of zebrafish embryos to chilling is probably related to the high lipid content of their yolk, and that the chilling injury can be reduced by reducing the amount of yolk in the embryos. Moreover, the low membrane permeability to cryoprotectants could also be tackled by introducing cryoprotectant by multi-punctures. The cryoprotectant toxicity study of the yolk-reduced embryos provided basic information for further studies. These findings from the present study may have significant implications in alleviating certain difficulties confronting fish embryo cryopreservation, as the suspected factors which complicate the cryopreservation of teleost embryos are mainly yolk related.

CHAPTER 6 DIFFERENTIAL SCANNING CALORIMETER STUDY ON CHARACTERISATION OF INTRAEMBRYONIC FREEZING AND CRYOPROTECTANT PENETRATION IN ZEBRAFISH EMBRYOS

6.1 Introduction

Although the high chilling sensitivity of embryos can be reduced by partial removal of their yolk at certain developmental stages such as prime-6 and high-pec (Chapter 5), it is necessary to study the kinetics of subzero chilling injury in yolk-reduced embryos before considering whether controlled slow cooling or vitrification should be employed for their cryopreservation (Mazur *et al.*, 1992). This is tantamount to asking about chilling sensitivity of unfrozen, supercooled embryos, and it needs the measurement of the limits of embryo supercooling. The study of intraembryonic supercooling and freezing would also provide important information for the design of effective protocols for the cryopreservation of fish embryos since supercooling and ice nucleation are crucial factors affecting the success of cryopreservation, especially when controlled slow cooling is applied. A previous study (Zhang *et al.*, 1993) on cryopreservation of zebrafish embryos using controlled slow cooling showed that ice formation within eggs was mainly responsible for the reduction of embryo survival. In order to maximise the survival of embryos during cryopreservation, the likelihood of intraembryonic ice formation must be minimised. Development of cryopreservation protocols for fish embryos involves the selection of optimal conditions, such as embryo developmental stages, pre-treatment cooling and post-thaw conditions, and any individual variation in these factors could influence the intraembryonic ice formation. To minimise the likelihood of ice formation in embryos, the possible mechanism of intraembryonic ice formation and the effect of above factors must be investigated.

Differential Scanning Calorimetry (DSC) has been used intensively to study intracellular ice formation (IIF) in a variety of cells (Franks and Bay, 1980; Rall *et al.*, 1982; Franks *et al.*, 1983; Bryant, 1995; Rasmussen *et al.*, 1997; El-Shakhs *et al.*, 1998). Recently, DSC has been employed to measure the nucleation temperature of intraembryonic water and the

amount of freezable water in insect embryos (Myers *et al.*, 1989; Schreuders *et al.*, 1996), and the freezing or melting point and the freezable water content in invertebrate organisms (Wharton and Ramlov, 1995; McAllen and Block, 1997; Wharton and Block, 1997). DSC measures the amount of heat absorbed or released by a sample as the temperature is changed. The instrument does this by determining the amount of electrical energy required to keep the temperature of the test sample equal to the temperature of the reference sample that contains no water. Ice formation in the embryo and its environs will produce a thermogram that shows an exothermic peak. Conversely, the melting of that ice produces an endothermic peak. The values of the onset temperature and peak temperature of the exotherm or endotherm can be obtained by analyses of the thermogram. Furthermore, from the area under the peak, which represents the heat energy that was absorbed or released during the phase change, the amount of frozen water within embryos can also be calculated.

DSC can also be used to estimate cryoprotectant permeation into embryos by measuring the depression of nucleation temperature since there is a linear relationship between the depression of the homogeneous nucleation temperature by low molecular weight solutes and the solute-induced freezing point depression (Rasmussen and MacKenzie, 1972). Understanding the cryoprotectant permeability of embryos is essential for developing either controlled slow cooling or vitrification procedures. The irregular shape and complex nature of the later stage embryos of zebrafish reduces the effectiveness and predictive value of conventional permeability studies involving the measurement of volumetric changes using light microscopy, and, to date, the permeability study of zebrafish has been limited to pre-6-somite stages. This study employed the DSC technique to study the cryoprotectant penetration in 6-somite, as well as prim-6 and yolk-reduced high-pec stage embryos.

The objective of this DSC study was to characterise intraembryonic freezing in intact, dechorionated, and yolk-reduced embryos with or without the treatment of cryoprotectants, and to estimate the cryoprotectant permeation into dechorionated or punctured embryos. The amount of freezable water and total water in an average embryo was also measured. In this study, 'intraembryonic' was defined as within a dechorionated embryo.

6.2 Results

6.2.1 Nucleation temperature of intraembryonic water

The nucleation temperature of intraembryonic water was evaluated with respect to the embryo developmental stage, dechorionation, partial removal of yolk, cooling rate, and the treatment of cryoprotectants.

6.2.1.1 Effect of embryo developmental stage and dechorionation

As illustrated in Fig. 6.1 and Fig. 6.2, intraembryonic freezing in both an intact and a dechorionated embryo was typically manifested in the DSC thermogram as a single peak or exotherm, and the peak in the dechorionated embryo shifted to a lower temperature. The freeze onset temperatures and peak temperatures for both intact and dechorionated embryos at 6-somite, prim-6 and high-pec stages are shown in Table 6.1. For intact embryos, the average exotherm began at -11.9, -15.6 and -20.5°C for prim-6, 6-somite and high-pec stage, respectively. The corresponding maximum peak temperatures were reached at -13.0, -16.0 and -21.3°C. The effect of developmental stage on the freezing temperatures was significant ($p < 0.01$). The later the developmental stage of intact embryos, the lower the

Table 6.1 Effect of dechorionation, developmental stage and partial removal of yolk on the nucleation temperatures of intraembryonic water of zebrafish.

Developmental stage	Freeze onset temperature (°C)		Freeze peak temperature (°C)	
	Intact	Dechorionated	Intact	Dechorionated
6-somite	-11.9 ± 1.5	-23.5 ± 0.8 *	-13.0 ± 1.2	-23.8 ± 0.8 *
prim-6	-15.6 ± 0.3	-18.7 ± 0.7 *	-16.0 ± 0.4	-19.0 ± 0.6 *
high-pec	-20.5 ± 0.1	-24.9 ± 0.8 *	-21.3 ± 0.2	-25.3 ± 0.7 *
yolk-reduced high-pec	—	-27.9 ± 0.4 †	—	-28.2 ± 0.4 †

Note: Values are averages ± standard errors (n = 3). The cooling rate was 10°C/min.
* Significant differences at 0.05 level between the values of dechorionated and that of intact embryos.
† Significant differences at 0.05 level between the values of dechorionated high-pec and that of yolk-reduced high-pec embryos.

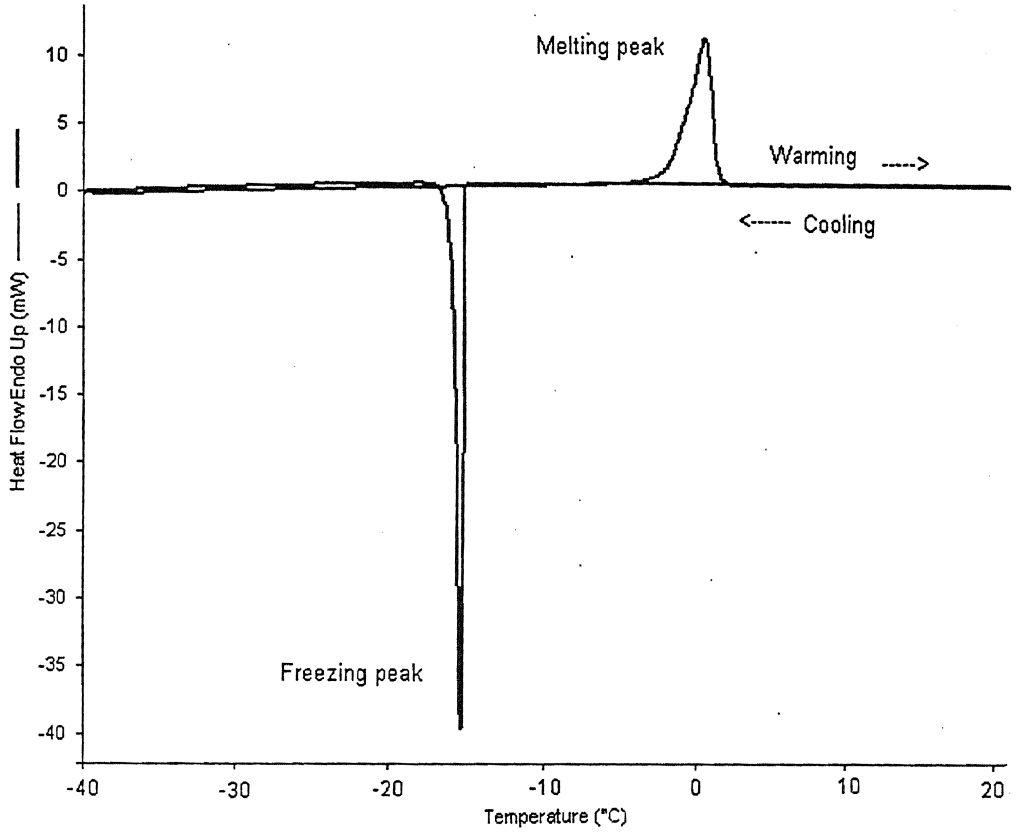


Fig. 6.1 DSC Thermogram from a typical intraembryonic ice formation experiments, showing the intraembryonic freezing peak and melting peak in an intact embryo at prim-6 stage. The freezing peak was initiated at - 15.1°C, with the peak maximised at -15.3°C.

nucleation temperatures. For dechorionated embryos, the exotherm began at -23.5, -18.7, and -24.9°C, with peaks at -23.8, -19.0, and -25.3°C, for 6-somite, prim-6 and high-pec stage, respectively. Dechorionated prim-6 embryos appeared to have higher nucleation temperatures than dechorionated embryos at either 6-somite ($p < 0.05$) or high-pec ($p < 0.01$) stage. Dechorionated embryos showed significantly lower ($p < 0.05$) freeze onset and peak temperatures than intact embryos for all the three stages. This indicated that intrembryonic freezing in intact embryos was induced by the freezing of perivitelline fluid between the plasma membrane and chorion.

The exothermic peak arising from the freezing of water during warming showed only a single smooth endotherm that occurred at about 0°C (Fig. 6 1-2). This demonstrated that the intact and dechorionated embryos remain supercooled to -10 to -21°C, and -17 to -25°C respectively, and only then undergo nucleation and freezing.

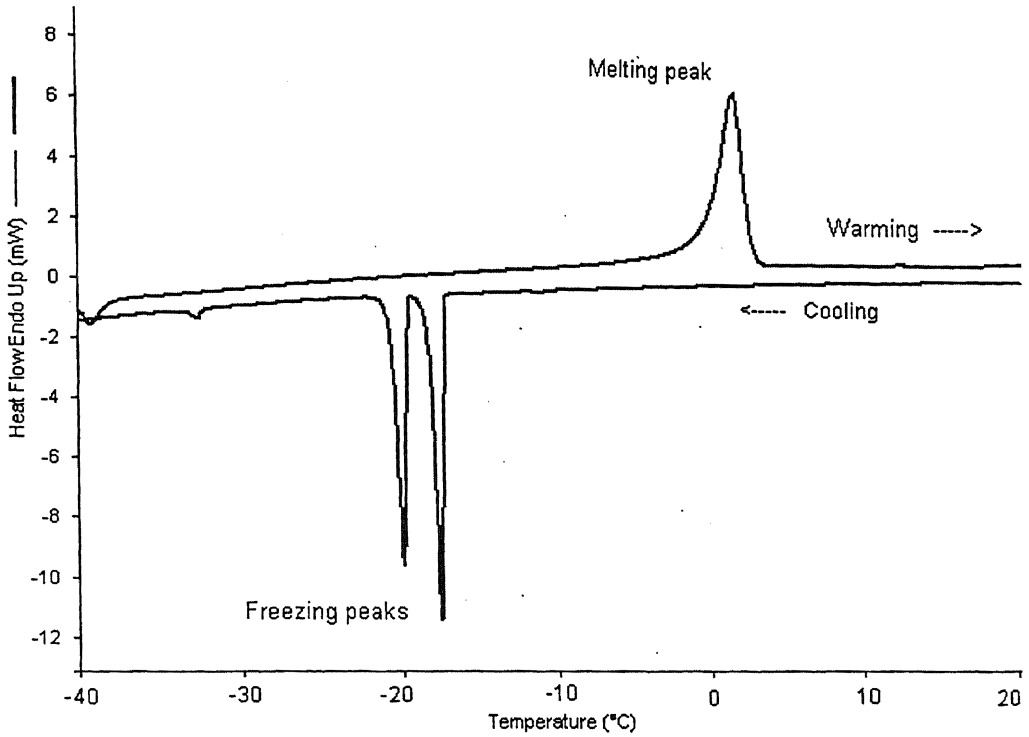


Fig. 6.2 DSC thermogram showing the intraembryonic freezing peak and melting peak in two dechorionated embryos at prim-6 stage. Two freezing peaks indicated that the nucleation of the two embryos occurred at different temperatures, with freeze onset temperatures at -17.2 and -19.6°C, and peak temperature at -17.5 and -19.9°C, respectively. The single melting peak represented that they melted at the same temperature around 0°C.

6.2.1.2 Effect of partial removal of yolk

After partial removal of yolk at prim-6 stage, the yolk-reduced high-pec embryos showed significantly ($p < 0.05$) lower freeze temperatures with an average onset at -27.9°C and an average peak at -28.2°C when compared with normal dechorionated high-pec embryos, with a freeze onset temperature at -24.9°C and a peak temperature at -25.3°C (Table 6.1). This indicated that partial removal of yolk helped embryos to supercool to a lower temperature.

6.2.1.3 Effect of cooling rate

The experiments were performed using both intact and dechorionated embryos at prim-6 stage. The freeze onset and peak temperatures were -15.3 ± 1.6 and -15.4 ± 1.6°C for intact embryos and -20.2 ± 0.9 and -20.3 ± 1°C for dechorionated embryos respectively when a -2°C/min cooling rate was applied. No significant differences were found in the temperature of intraembryonic ice formation between the two different cooling rates tested of 2°C/min

and 10°C/min (see Table 6.1), suggesting that the intraembryonic freezing was independent of the cooling rate employed in this study.

6.2.1.4 Effect of cryoprotectants

Methanol The treatment with 2 M methanol (2 h at room temperature) significantly ($p < 0.05$) decreased the nucleation temperatures of dechorionted 6-somite embryos from -23.5 to -27.8 °C (onset temperature) and -23.8 to -28.0°C (peak temperature) although it did not alter ($p > 0.05$) the freezing temperatures of dechorionated prim-6 or yolk-reduced high-pec stage embryos (Table 6.2). However, following 30 min exposure to 2 M methanol, punctured prim-6 embryos also showed significantly ($p < 0.05$) lower freeze onset and peak temperatures (Fig. 6.3) than dechorionated prim-6 embryos with or without 2 M methanol treatment. These results demonstrated that methanol penetrated the 6-somite embryos, but not those at prim-6 or high-pec stages. However, methanol permeated into the punctured prim-6 embryos.

Propylene glycol Table 6.3 shows the effect of the treatment of 1 M PG on the temperature of intraembryonic freezing of zebrafish embryos at 6-somite and prim-6 stages. After 2 h exposure to 1 M PG, the nucleation temperatures of dechorionated 6-somite, prim-6 or

Table 6.2 Effect of 2 M methanol on the nucleation temperatures of intraembryonic water of zebrafish.

Developmental stage	Freeze onset temperature (°C)		Freeze peak temperature (°C)	
	Dechorionated	Punctured	Dechorionated	Punctured
6-somite	-27.8 ± 0.7	—	-28.0 ± 0.7 $^{\$}$	—
prim-6	-20.3 ± 1.7	-26.8 ± 1.4 *†	-20.6 ± 1.7	-27.1 ± 1.4 *†
Yolk-reduced high-pec	-30.9 ± 1.3	—	-31.2 ± 1.2	—

Note: Values are averages ± standard errors (n = 3).

* Significant differences at 0.05 level between the value of punctured and that of dechorionated embryos.

⇑ Significant differences at 0.05 level when compared with the value of corresponding dechorionated embryos without the treatment of cryoprotectant (see Table 6.1).

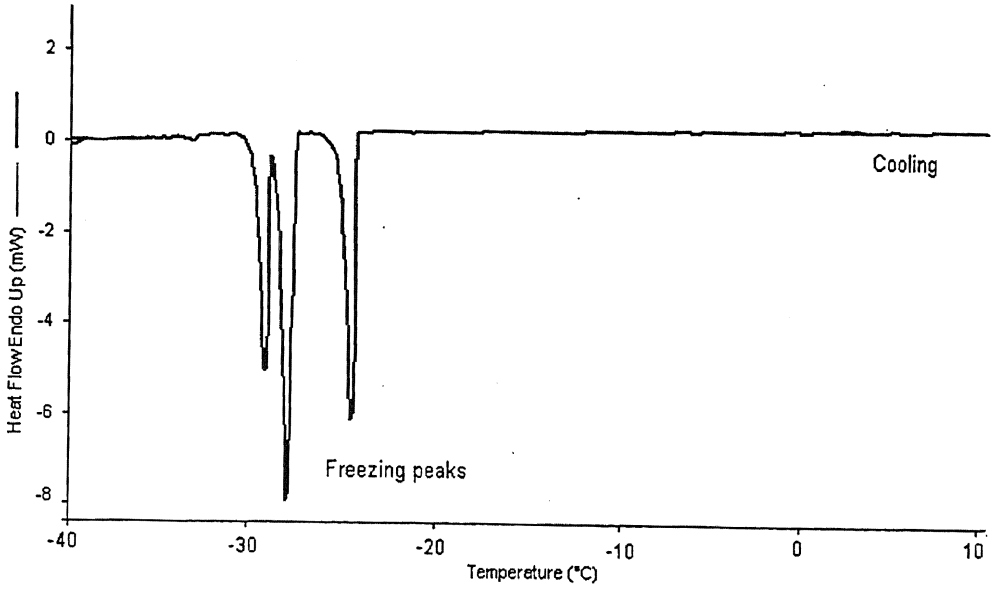


Fig. 6.3 DSC thermogram showing the cooling trace of three punctured embryos at prim-6 stage treated with 2 M methanol for 30 min at room temperature. The three separate freezing peaks indicated that the freezing of the three embryos occurred at different temperatures.

Table 6.3 Effect of 1 M PG on the nucleation temperatures of intraembryonic water of zebrafish.

Developmental stage	Freeze onset temperature (°C)		Freeze peak temperature (°C)	
	Dechorionated	Punctured	Dechorionated	Punctured
6-somite	-23.6 ± 1.2	-30.4 ± 1.6 *†	-23.8 ± 1.2	-30.6 ± 1.5 *†
prim-6	-20.2 ± 1.9	-27.8 ± 0.8 *†	-20.5 ± 1.9	-28.1 ± 0.8 *†
yolk-reduced high-pec	-26.7 ± 0.5	—	-27.1 ± 0.5	—

Note: Values are averages ± standard errors (n = 3).

* Significant differences at 0.05 level between the value of punctured and that of dechorionated embryos.

† Significant differences at 0.05 level when compared with the value of corresponding dechorionated embryos without the treatment of cryoprotectant (see Table 6.1).

yolk-reduced high-pec embryos were similar ($p > 0.05$) to those without cryoprotectant treatment, suggesting that all these embryos were impermeable to PG. However, treatment with 1 M PG (30 min at room temperature) shifted the nucleation temperatures of punctured 6-somite or prim-6 embryos to significantly lower ($p < 0.05$) temperatures compared to those of corresponding normal dechorionated embryos with or without PG treatment. The results demonstrated that multi-puncture resulted in permeation of PG into dechorionated 6-somite or prim-6 embryos.

The depression of homogeneous nucleation temperature by cryoprotectants in relation to their concentration can be calculated according to Rasmussen and MacKenzie's findings (1972) that low molecular weight solutes depress the homogeneous nucleation temperature by about 1.8 times the solute-induced depression of freezing point or about 3.3°C (1.8×1.86°C/osm) for each unit increase in solution osmolality. The effect of solutes on the temperature of heterogeneous nucleation is similar to the effect the solutes have on homogeneous nucleation (Reid *et al.*, 1985). Table 6.4 shows the comparison of the calculated homogeneous nucleation temperature depression by 2 M methanol or 1 M PG and the observed nucleation temperature depression in dechorionated or punctured 6-somite and prim-6 embryos which were treated with either 2 M methanol or 1 M PG. In dechorionated 6-somite embryos, the observed depression of nucleation temperature (4.3°C)

Table 6.4 Calculated and observed nucleation temperature depression by 2 M methanol and 1 M PG in dechorionated or punctured zebrafish embryos

Cryoprotectant	Observed nucleation temperature depression (°C)[a]				Calculated nucleation temperature depression (°C)[b]
	6-somite		prim-6		
	Dechorionated	Punctured	Dechorionated	Punctured	
2 M methanol	4.3	—	1.6	8.1	7.3
1 M PG	0.1	6.9	1.5	9.1	4.0

Note: a. The data was calculated by the freeze onset temperatures of cryoprotectant treated embryos minus those of dechorionated embryos without cryoprotectant treatment (absolute values).

b. The data was determined by 3.3 × cryoprotectant osmolality (osm/kg), which are 2.2 and 1.2 osm/kg for 2 M methanol and 1 M PG solution, respectively.

was considerably lower than the calculated value of 7.3 for 2 M methanol. This indicated that after 2 h exposure to 2 M methanol at room temperature, the methanol concentration in the embryos was considerably less than 2 M, suggestive of limited permeation of 2 M methanol into dechorionated 6-somite embryos. On the other hand, the observed depression of nucleation temperature by both cryoprotectants in punctured embryos was higher than those of calculated value, indicating that punctured embryos became much more permeable to methanol and PG than dechorionated embryos.

6.2.2 Intraembryonic water content

6.2.2.1 Amount of freezable water

The amount of heat released during freezing is proportional to the mass of intraembryonic ice. The enthalpy (area of the exotherm) of the phase transition during intraembryonic freezing in individual embryos was therefore used to estimate the amount of freezable water in the embryos. The heat of fusion (L_f) decreases with the reduction of temperature and therefore, to calculate the mass of ice from enthalpy, the values of L_f should be corrected according to the temperature at which the freezing occurs rather than the value of L_f at 0°C (79.9 cal/g, or 333.5 J/g). The corrected values of L_f can be obtained using a refinement of Kirchhoff's law by the following equation (Mazur, 1963a):

$$L_f = L_f^0 + 9.080T - 0.02649T^2 + 0.000216T^3,$$

where L_f^0 is the heat of fusion at 0°C (79.9 cal/g) and T is the Celsius temperature. The temperature used in this calculation was the freeze peak temperature of each embryo. The calculated amount of freezable water per dechorionated embryo was 88.3 ± 5.0, 99.9 ± 12.5 and 97.7 ± 1.6 μg for 6-somite, prim-6 and high-pec stages, respectively (Table 6.5). The differences between them were not significant ($p > 0.05$). In yolk-reduced high-pec embryos, the amount of freezable water per embryo was 73.8 ± 2.8 μg.

Table 6.5 Amount of freezable water and total water per dechorionated zebrafish embryo

Developmental stage	Amount of freezable water (μg)	Amount of total water (μg)	Total weight of embryo (μg)
6-somite	88.3 ± 5.0	115.0 ± 3.0	195.7 ± 1.9
prim-6	99.9 ± 12.5	113.7 ± 1.8	194.0 ± 1.2
high-pec	97.7 ± 1.6	108.0 ± 3.8	188.0 ± 3.2
yolk-reduced high-pec	73.8 ± 2.8**	77.3 ± 4.3**	96.0 ± 2.5**

Note: Values are averages ± standard errors (n = 3).

** Significant differences at 0.01 level between the value of yolk-reduced high-pec and that of other embryos.

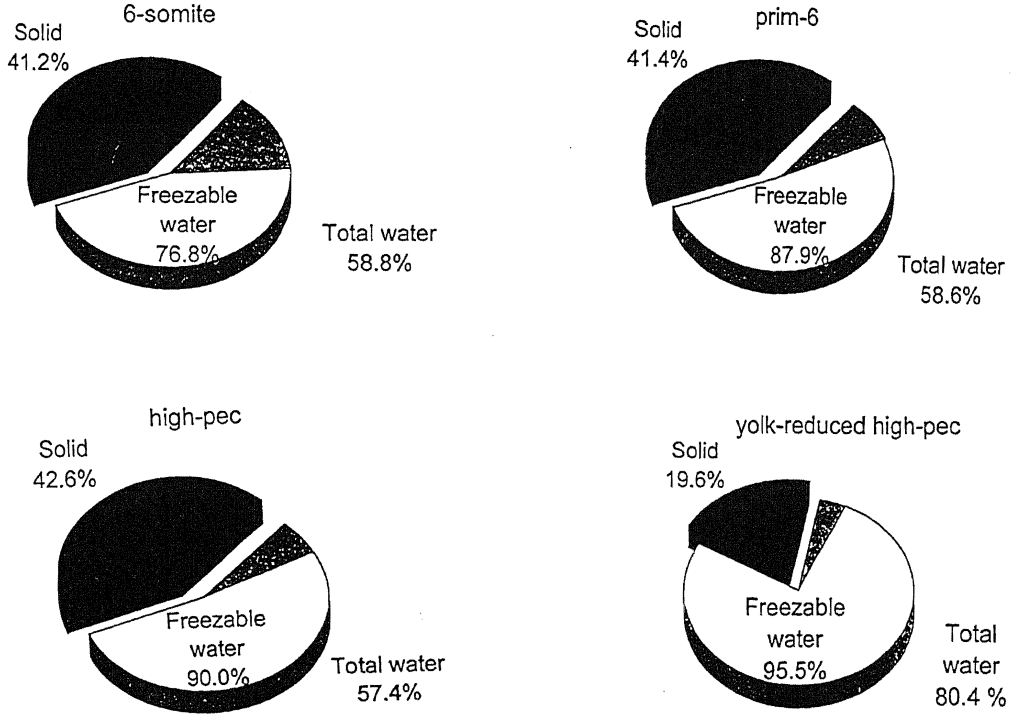


Fig. 6.4 Percentage of total water and freeezable in dechorionated embryos at different stages and yolk-reduced high-pec embryos.

6.2.2.2 Amount of total water

As shown in Table 6.5, the amounts of total water per dechorionated embryo for 6-somite, prim-6 and high-pec stages were 115.0 ± 3.0, 113.7 ± 1.8 and 108.0 ± 3.8 μg, respectively. The corresponding fully hydrated weights per embryo were 195.7 ±1.9, 194.0 ± 1.2 and 188.0 ± 3.2 μg. These amounts of water constituted 58.8 ± 2.1, 58.6 ± 0.6, 57.4 ± 1.0% of the total mass of the fully hydrated embryos at 6-somite, prim-6 and high-pec stage, respectively. The differences between these stages were not significant ($p > 0.05$). However, for yolk-reduced high-pec embryos, the percentage of total water of 80.4 ± 2.6% was significantly higher ($p < 0.01$) than dechorionated high-pec and other stage embryos. The comparisons of the percentage of total and freeezable water in dechorionated embryos at different stages are shown in Fig. 6.4. The fractions of the total water that were freezable (column 2 versus column 3 in Table 6.5) were 76.8, 87.9, 90.0 and 95.5% for 6-somite, prim-6, high-pec and yolk-reduced high-pec embryos, respectively.

6.3 Discussion

6.3.1 Mechanism of intraembryonic freeezing

In intact zebrafish embryos, there are two distinct membranes, namely the outer chorionic membrane (chorion) and the inner plasma membrane (Hisaoka, 1958). Between these two membranes there is a perivitelline space which is filled with a perivitelline fluid and within the plasma membrane are the yolk and blastoderm. The DSC thermogram of an intact embryo showed only a single smooth freeze peak despite there being three compartments within the embryo. The results indicated that these three compartments were frozen simultaneously during cooling. The fact that the freeze temperatures of dechorionated embryos shifted to significantly lower temperatures demonstrated that the freezing of perivitelline fluid triggers the ice formation inside the yolk and blastoderm during the cooling, therefore intraembryonic freezing within intact embryos is seeding by extraembryonic ice.

The molecular basis of seeding is not well understood. Mazur (1977) proposed that intracellular ice formation (IIF) occurs as a consequence of ice crystal growth through

crystals having a sufficiently small radius of curvature. Steponkus *et al.* (1983) observed mechanical failure of the plasma membrane of isolated plant protoplasts preceding IIF, which suggests that the membrane was destabilised prior to ice propagation into cells. Muldrew and McGann (1990) hypothesised that the plasma membrane is damaged at a critical gradient in osmotic pressure across the membrane, and IIF occurs as a result of this damage. Recently, Toner *et al.* (1990, 1993) have proposed that IIF is catalysed heterogeneously by the plasma membrane in the presence of external ice, a mechanism referred to as surface-catalysed nucleation.

With dechorionated embryos, in order to preclude seeding by external ice, all extraembryonic water was removed and the embryos were suspended in silicone oil, which was used as carrier liquid in a few nucleation studies (Rasmussen and MacKenzie, 1972; Felix *et al.*, 1983). A pilot experiment showed that during the cooling and warming no thermal events occurred in the silicone oil. Mild fixation by glutaraldehyde (3%, 5 min at room temperature) was applied to toughen the surface of embryos before extraembryonic water removal. The fixation did not cause any dehydration of 6-somite embryos (Hagedorn *et al.*, 1997), and no extra transitions were found in the thermogram of the fixed embryos. It was therefore believed that glutaraldehyde fixation did not affect the DSC thermogram analysis.

The possibility of seeding by extraembryonic ice in dechorionated embryos is precluded by two characteristics of the observed intraembryonic freezing: (a) the nucleation temperatures of the embryos at a specific stage fell within a narrow range ($\pm < 1.4$°C); and (b) the nucleation temperatures were independent of the cooling rate employed. Both these factors are typical of homogeneous or heterogeneous nucleation in highly purified aqueous solutions in cells (Franks *et al.*, 1983, Rasmussen and MacKenzie, 1972). However, it remains to be determined whether intraembryonic ice formation in dechorionated embryos is the consequence of homogeneous or heterogeneous nucleation. The homogeneous nucleation temperature of a 1 μm droplet of pure water is -39°C (Thomas and Staveley, 1952), and increases by approximately 2°C for each 10-fold increase in droplet diameter (Wood and Walton, 1970). Solutes depress the homogeneous nucleation temperature by 3.3°C for each unit increase in solution osmolity (Rasmussen and MacKenzie, 1972). The isosmotic value of the zebrafish embryo cytoplasm is unknown (Hagedorn *et al.*, 1997). If the isosmotic value of 0.3 osm (the value of most animal cells) is used, the calculated

the isosmotic value of 0.3 osm (the value of most animal cells) is used, the calculated temperature of homogeneous nucleation of dechorionated zebrafish embryos should be about -35°C since the average freezable water determined by DSC in dechorionated zebrafish embryos is approximately 95 μg, equivalent to a droplet of 566 μm. This temperature is more than 10°C below the nucleation temperatures of dechorionated embryos determined by DSC. Therefore, it is more likely that heterogeneous nucleation is the cause of the freezing of dechorionated zebrafish embryos. Heterogeneous nucleation relies on the presence of intracellular nucleating agents to catalyse ice crystallisation at a higher temperature. Evidence for intracellular nucleators has been provided by several studies (Franks and Bray, 1980; Franks *et al.*, 1983; Myers *et al.*, 1989) although there are contradictory reports (Rasmussen *et al.*, 1975; Burke *et al.*, 1976). The nucleating agents in the embryos become effective in the temperature range of -17 to -26°C, depending on the developmental stages.

It is difficult to explain the fact that dechorionated embryos at prim-6 stage have significantly higher nucleation temperatures than the other two stages. For intact embryos, the lower freezing temperatures of the later stages may be attributable to increasing solute concentration in the perivitelline fluid by the release of metabolic products from the developing embryo.

For yolk-reduced high-pec embryos, intraembryonic freezing shifted to lower temperatures compared to dechorionated (control) high-pec stage embryos, but the temperatures were still well above the level for homogeneous nucleation. Again heterogeneous nucleation is probably the cause of intraembryonic ice formation of these embryos although the heterogeneous nucleating agents in these embryos become effective at lower temperatures. Considering that more than half of the yolk was removed at prim-6 stage, the yolk-reduced high-pec embryos contained little yolk content after 24 h development following the removal of yolk. The heterogeneous nucleating agents, which induce the heterogeneous nucleation of normal dechorionated embryos, may be mainly present in the yolk. Yolk granules, which consist of crystalline carotenopholipoprotein, have been suspected to play a role in the ice nucleation of penaeid prawn larvae (Arun and Subramoniam, 1997). Although there are two different compartments of yolk and blastoderm in dechorionated and yolk-reduced embryos, the nucleation of one compartment is likely to induce the nucleation of the other. Ice nucleation through junction between cells

has been explained for the induction of intracellular ice between adjacent cells (Berger and Uhrik, 1996; Acker *et al.*, 1999).

Whilst no other comparable intraembryonic freezing temperature data can be found for fish embryos, a comparison of IIF temperatures with other species in the presence and absence of extracellular ice is shown in Table 6.6. The results from the present study are consistent with the common observation that IIF occurs above -15°C only in the presence of extracellular ice and the temperatures of IIF are much lower in the absence of external ice.

Table 6.6 Intracellular ice formation of isolated cells, eggs or larvae in the presence and absence of extracellular ice (modified from Toner, 1993 and Wang, 1998)

Cell type	Temperature of IIF (°C)		Method of preventing external ice	References
	External ice present	External ice absent		
Yeast	-10 to - 15	-40 to -47	in oil	Mazur, 1961, 1965
See urchin eggs	-4 to -8	< -15	suspension in oil; supercooling	Asahina, 1961; Hubel *et al.*, 1987
Spirogyra	-7.7	< -12.4	supercooling	Morris and McGrath, 1981
rainbow trout eggs	> -7.5	-18 to -20	in paraffin oil	Harvey and Ashwood-Smith, 1982
Erythrocytes	-10 to -15	< -35	in oil	Diller,1975; Mathias *et al.*, 1984
Mouse oocytes	-12.5	< -20	supercooling	Toner *et al.*, 1991
Drosophila melanogaster embryos	-10.2	-28.5	eggcase	Myers *et al.*, 1989
Anopheles gambiae embryos	< -29.3	< -28.4	in mineral oil	Schreuders *et al.*, 1996
Nematode *Panagrolaimus davidi*	-7	-26.5	in paraffin	Wharton and Block, 1997
Nereis virens larvae	< -18	<-31.2	in paraffin	Wang, 1998
Zebrafish *Danio rerio* embryos	-10.1 to -20.6	-17.2 to -26.2	in silicone oil	Present study

This suggests that seeding by the extracellular ice is the common mechanism of IIF. However, studies on *Anopheles* mosquito embryos (Schreuders *et al.,* 1996) have shown that these embryos supercooled some 20°C below the temperature of external ice formation and supercooled as much in the presence of external ice as its absence. This indicates that external ice crystals are unable to penetrate the embryos to seed the supercooled water within. Most of the barrier to ice nucleation in *Anopheles* embryos lies in the inner vitelline membrane, which possesses low or nearly undetectable permeability to liquid water (Schreuders *et al.,* 1996). For zebrafish embryos, the water permeability of their membranes is also low (Hagedorn *et al.*, 1997; Zhang and Rawson, 1997, 1998), particularly at a later stage like high-pec, when they become increasingly melanized (Kimmel *et al.*,1995), possibly involving protein crosslinking, which may augment the barrier. However, results of this study suggest that external ice crystals can pass through the permeability barriers in zebrafish embryos for all the tested developmental stages.

6.3.2 Depression of nucleation temperature and cryoprotectant permeability

The depression of heterogeneous nucleation temperature by cryoprotectants has been reported in several studies (Rall *et al.*, 1983; Reid *et al.*, 1985; Myers *et al.*, 1989). Rall *et al.* (1983) pointed out the possibility that the cryoprotectants may reduce the ability of heterogeneous nucleators to catalyse the formation of stable ice nuclei by altering the thermodynamic stability of ice clusters in a manner comparable to that proposed by Rasmussen and MacKenzie (1972) for the effects of solutes on homogeneous nucleation. Therefore, the nucleating agents in the cells or embryos become less effective in the presence of cryoprotectants and then the nucleation temperatures are depressed.

The linear relationship found by Rasmussen and MacKenzie (1972) between the depression of the homogeneous nucleation temperature by the low molecular weight solutes and the solute-induced freezing point depression was applied to estimate the cryoprotectant permeability. Although using the cooled plate and droplet techniques, Reid *et al.*, (1985) found that the effect of solutes on the temperature of heterogeneous nucleation of ice in aqueous solution and in aqueous silver iodide was similar to the effect the solutes have on homogeneous nucleation; in this study, the effect of cryoprotectants on the temperatures of

heterogeneous nucleation was actually more pronounced than the observed nucleation temperature depression by 2 M methanol and 1 M PG in punctured embryos. Myers *et al.* (1989) also reported a similar observation with *Drosophila* embryos suspended in 1 to 2 M ethylene glycol and the heterogeneous nucleation temperatures were found to be decreased approximately 12°C for each increment in the molar concentration of ethylene glycol. Rall *et al.* (1983) found that heterogeneous nucleation temperatures of mouse embryos decreased from -10 to -15°C in saline alone to between -38°C and -44°C in 1 to 2 M glycerol and DMSO solutions, which were also larger than the calculated values of homogeneous nucleation temperature depression by the two cryoprotectants.

Although the concentration of cryoprotectants in embryos can not be determined quantitatively according to the depression of heterogeneous nucleation temperature by cryoprotectants for the reasons outlined above, the permeation of cryoprotectants into embryos can be qualitatively evaluated by comparing the observed temperature depression of heterogeneous nucleation and the calculated temperature depression of homogeneous nucleation. The results of depression of nucleation temperatures suggested that 2 M methanol can penetrate dechorionated 6-somite zebrafish embryos but the penetration is limited. These findings are in good agreement with other studies on the permeability of dechorionated prim-6 zebrafish embryos using magnetic resonance microscopy and electron spin resonance techniques (Hagedorn *et al.*, 1996) and measurement of volumetric changes (Zhang and Rawson, 1998). However, 2 M methanol was found to be unable to permeate into dechorionated embryos at later stages such as prim-6 and high-pec. This may be due to a new permeability barrier, epithelia, developed around the embryos at these stages (Kimmel *et al.*, 1995). Little or no depression of nucleation temperatures was observed when dechorionated embryos at all stages were exposed to 1 M PG. It is therefore believed that these embryos were not permeable to PG. Hagedorn *et al.* (1996) also found that PG exhibited little or no permeation into the yolk of 3-somite stage embryos although it appeared to penetrate the blastoderm of the embryos. However, multi-punctures of yolk resulted in the permeation of both methanol and PG into prim-6 or 6-somite embryos.

The present DSC studies showed that after 2 M methanol treatment, dechorionated prim-6 embryos freeze at about -20°C. This is generally consistent with results from a previous study by Zhang *et al.* (1993). By direct visual observation of the responses of embryos during cooling, they found that with a cooling rate of 0.3°C/min, ice formation (whitening)

within the egg occurred in approximately 50, 70 and 90% intact heart-beat (prim-6) embryos at -20, -25 and -30°C, respectively after 1 h exposure to 2 M methanol at room temperature. Different procedures and assessment methods applied in their study may account for the lower temperatures of ice formation within the embryos. The combination of slow cooling (0.3°C/min) and the introduction of external ice nucleation (seeding) at -7.5°C in their procedures probably enhanced the dehydration of embryos and thus resulted in lower nucleation temperatures as a consequence of the increase in the concentration of solutes in the embryos. In their study, the ice formation within embryos was visually inspected at 5°C intervals rather than at consecutive temperatures, and it may cause a maximum of 5°C error (lower than the actual nucleation temperature).

As discussed above, the presence of external ice seeds the intraembryonic freezing in intact embryos. However, an apparently contradictory observation is that embryos were not seeded by the external ice nucleation induced at above -10°C when they were suspended in cryoprotectant solution (Zhang *et al.*, 1993). An explanation could be that the presence of cryoprotectants such as methanol reduce the effectiveness of seeding by extracellular ice. The higher the concentration of the cryoprotectants, the greater will be the fraction of the solution remaining unfrozen at a given subzero temperature (Rall *et al.*, 1978; Mazur *et al.*, 1981), and the lower will be the likelihood that ice will come in direct contact with the embryo membrane. Based on the hypothesis that intracellular freezing by seeding is a consequence of prior membrane change at a critical gradient in osmotic pressure across the membrane (Muldrew and McGann, 1990), another possible explanation is that cryoprotectants might be able to prevent changes in membrane architecture that would permit the passage of extracellular ice crystal at higher temperature.

6.3.3 Amount of freezable water versus amount of total water

The total mass of water was determined gravimetrically being 108 - 115 μg/per embryo, and was independent of developmental stage. Water constitutes 57.4 to 58.8% of the total weight of dechorionated embryos. These values are well in agreement with the 58% reported by Hagedorn *et al.* (1997) using magnetic resonance microscopy and electron spin resonance techniques to determine water content in dechorionated 6-somite embryos, although they also reported a larger value (74%) of water content in the same stage embryos using the similar wet/dry-weight measurement method. They explained this discrepancy on

the basis that their wet/dry-weight measurements overestimated the water content due to the excess unremovable water adhering to the outer surface of the embryos before weighing (Hagedorn *et al.*, 1997). The total percentage of water in dechorionated zebrafish embryos is considerably less than those of other embryos or eggs of yolk-laden species reported so far (Table 6.7).

The amount of freeezable water in dechorionated embryos was estimated from the area under the exotherm during cooling. The corrected L_f values were used to calculate the mass of water from the heat released during freezing due to the decrease of L_f with temperature. The calculated fraction of total water that is freezable was between 76.9 and 90.0%. Later stage embryos seemed to have a larger fraction of freezable water although the differences in the amount of freezable water between stages were not statistically significant. These values are comparable to those of *Anopheles gambiae* embryos, but *Drosophila* embryos have been reported to have relatively less freezable water (Table 6.7).

Table 6.7 Percentage of freezable water and total water in embryos or eggs of some yolk-laden species

Species	Freezable water (%)[a]	Total water (%)	References
Insects			
Drosophila melanogaster embryos	68	76.2	Myers *et al.*, 1987
Anopheles gambiae embryos	85 - 91	72.7 - 75.5	Schreuders *et al.*, 1996
Fish			
Zerafish *Danio rerio* embryos	76.8 - 90.0	57.4 - 58.8	Present study
Rainbow trout eggs	—	66.2	Blaxter, 1969
Sardine eggs	—	70.7	Blaxter, 1969
Coho salmon eggs	—	61.0	Hardy *et al.* 1984

Note: a. The percentage of the total water that is freezable.

6.3.4 Implications for cryproservation

This is the first report of the intraembryonic freezing temperatures determined by DSC for zebrafish. Knowledge of the limits of embryo supercooling allows the study of the kinetics of subzero chilling injury in the yolk-reduced embryos independent of intraembryonic ice formation as performed with *Drosophila* embryos (Mazur *et al.*, 1992). Such information will be crucial to make a decision on whether or not slow cooling could be applied to the cryopreservation of the embryos. Because external ice can induce intraembryonic ice formation, it is important to remove all extraembryonic water when studying the subzero chilling sensitivity of the embryos. As observed in this study, the yolk-reduced high-pec embryos remained supercooled to below -27°C, and it is therefore possible to determine the kinetics of chilling injury down to that temperature.

To achieve successful cryopreservation, embryos must survive cooling to below -100°C and in the process of cooling they must not freeze intracellularly or intracellular freezing must be kept to minimum, so their survival is not affected. In theory, slow cooling the embryos to allow freezable intraembryonic water to flow out of the embryo in combination with cryoprotectant permeation into embryos to depress the nucleation temperature could avoid such freezing if the reduction in chilling injury was sufficient to permit the application of such slow cooling. However, the yolk-reduced embryos appeared to be impermeable to both cryoprotectants tested although further study on the permeability of the embryos to water and other cryoprotectants might be warranted. Unfortunately, unlike earlier stage embryos such as 6-somite and prim-6, increasing the cryoprotectant permeation into the yolk-reduced high-pec embryos by multi-punctures of the yolk is ineffective since the yolk constitutes a very small fraction of the whole embryo. Other approaches to increasing their permeability still need to be developed even if slow cooling is applicable to these embryos.

The depression of nucleation temperature by 2 M methanol or 1 M PG in punctured prim-6 and 6-somite embryos indicated that these two embryo stages became permeable to cryoprotectants after multi-punctures. This may have important implications for cryopreservation of zebrafish embryos. Cryoprotectant penetration may modify the high chilling sensitivity of zebrafish embryos at these stages, but previous studies with *Drosophila* embryos showed that cryoprotectants made little contribution to mitigating the chilling injury in permeabilised embryos (Leibo *et al.*, 1988; Mazur *et al.*, 1992). Therefore,

a vitrification cryopreservation strategy may still be preferable if the embryos could tolerate vitrification concentrations of cryoprotectants.

This study also presents a new method for studying the membrane permeability of these embryos. Although the concentration of cryoprotectants in embryos can not be quantitatively determined, the strong agreement between the DSC study and other reported methods on cryoprotectant permeability of 6-somite zebrafish embryos showed the reliability of this DSC approach. Moreover, the DSC method has an advantage over conventional morphometric measurements by light microscopy in determining the permeability of later stage fish embryos, since the latter techniques are difficult to apply because of the irregular shape and complex nature of these stage embryos.

6.4 Summary

DSC was applied for the first time to determine the nucleation temperatures of intraembryonic water and cryoprotectant penetration in zebrafish embryos in the present study. The effect of embryo developmental stage, dechorionation, partial removal of yolk, cooling rate and the treatment of cryoprotectants on the temperatures of intraembryonic freezing were investigated. Embryo stages have been found to have significant effect on the nucleation temperatures of intact embryos. The later the developmental stage of intact embryos, the lower the temperatures of intraembryonic freezing. Dechorionated embryos showed significantly lower nucleation temperatures when compared with corresponding intact embryos. Partial removal of yolk shifted the nucleation temperatures of high-pec stage embryos to significantly lower temperatures. No significant differences were found in the temperatures of intraembryonic freezing between the two different cooling rates of 2 and 10°C/min.

The effect of cryoprotectant treatment on the nucleation temperatures of intraembryonic water varies among different embryo stages and different cryoprotectants. The treatment of 2 M methanol significantly decreased the nucleation temperatures of dechorionted 6-somite embryos whilst no temperature decrease was observed for prim-6 or yolk-reduced high-pec embryos. However, after 30 min exposure to 2 M methanol, punctured prim-6 embryos showed significantly lower temperatures of intraembryonic freezing. Treatment with 1 M

PG did not significantly affect the nucleation temperatures of dechorionated 6-somite, prim-6 or yolk-reduced high-pec embryos. The nucleation temperatures of punctured 6-somite or prim-6 embryos, however, were decreased significantly after exposure to 1 M PG for 30 min.

The amount of freezable water in single embryos was also calculated from the DSC measurements and the total water contents were determined gravimetrically. The calculated amount of freezable water per dechorionated embryo was 88.3 ± 5.0, 99.9 ± 12.5, 97.7 ± 1.6 and 73.8 ± 2.8 μg for 6-somite, prim-6, high-pec and yolk-reduced high-pec stage embryos, respectively. The corresponding amounts of total water were 115.0 ± 3.0, 113.7 ± 1.8, 108 ± 3.8 and 77.3 ± 4.3 μg, respectively. They constitute 58.8, 58.6 and 57.4% of the corresponding total weight of dechorionated embryos and 80.4% of full mass of yolk-reduced high-pec embryos. These differences between stages were not significant except in yolk-reduced embryos.

The results suggested that in intact embryos, intraembryonic freezing was seeded by the external ice in the perivitelline fluid, and in dechorionated embryos (in the absence of external water), intraembryonic freezing was more likely a consequence of heterogeneous nucleation. The effectiveness of the heterogeneous agents appeared to be reduced in the yolk-reduced embryos. Methanol was demonstrated to show a limited degree of penetration into prim-6 stage embryos, but it did not penetrate later stage embryos such as prim-6 and yolk-reduced high-pec. No PG permeation was observed for embryos at all stages. However, multi-punctures resulted in the permeation of both cryoprotectants into prim-6 embryos and PG permeation into 6-somite embryos. There findings may have important implication in overcoming the problem associated with low membrane permeability of zebrafish embryos to cryoprotectants.

CHAPTER 7 CONCLUSIONS

7.1 Reiteration of Aims

Although the cryopreservation of embryos of most domestic species has become a routine procedure (Friedler, 1988; Palasz and Mapletoft, 1996), the cryopreservation of fish embryos presents significant difficulties which have not been overcome using current cryopreservation technology. The major factors responsible for this failure have been identified as: (1) low permeability of embryo membranes to water and cryoprotectants; (2) their high chilling sensitivity; and (3) the two compartment nature of the embryo with a high yolk content. The aim of this investigation was to use zebrafish as a model system to study these limiting factors with a view to ultimately developing an effective cryopreservation technique for fish embryo preservation by overcoming the limitations.

To achieve this aim, four main areas were investigated with zebrafish embryos: (1) a vitrification of the embryos using methanol as the cryoprotectant, (2) the effects of cooling rate and developmental arrest on the chilling sensitivity of the embryos, (3) the effect of partial removal of yolk on chilling injury and cryoprotectant toxicity in these embryos, and (4) a differential scanning calorimetry study on the characterisation of intraembryonic freezing and cryoprotectant penetration in these embryos.

7.2 Review of the Main Findings

7.2.1 Methanol toxicity to zebrafish embryos

Methanol was chosen as the cryoprotectant for vitrification as it has been found to be the only cryoprotectant to permeate zebrafish embryos quickly (Zhang and Rawson, 1996a; Hagedorn *et al.*, 1996), and also to have a relatively low toxicity (Zhang and Rawson, 1993). However, there was no data available on the toxicity of methanol at a vitrifying concentration to zebrafish embryos. The lowest apparent vitrification concentration was found to be 10 M at 1 μl volume. Because the volume of an intact zebrafish embryo is about

0.5 μl, the toxicity of 10 M methanol at 0°C to zebrafish embryos was therefore investigated and the toxicity was found to be affected by the following factors:

(a) dilution method for the removal of cryoprotectant: 10 M methanol was shown to be less toxic to intact 6-somite embryos following one-step embryo medium dilution;
(b) embryo stages: later stage (50%-epiboly to prim-6) embryos appeared to be less sensitive to 10 M methanol than early stage (1-cell and 64-cell) embryos;
(c) dechorionation: dechorionated embryos seemed to be more sensitive to 10 M methanol toxicity than intact embryos.

These results confirmed previous findings (Zhang, 1994) on stage-dependent sensitivity to cryoprotectants and the negative effect of dechorionation on the sensitivity of zebrafish embryos. The study also revealed that the dilution method has significant effect on cryoprotectant toxicity to zebrafish embryos. These findings provided the basic information for the design of a vitrification protocol by using methanol. Based on the results obtained above, intact embryos and a one-step embryo medium dilution method were used, and stage-specific pretreatment procedures were designed in the following vitrification study.

7.2.2 Vitrification of zebrafish embryos

Vitrification has been successfully applied to the cryopreservation of embryos of more than ten animal species. Previous unsuccessful attempts at vitrification of zebrafish embryo had been performed only with two embryo developmental stage (6-somite and prim-6) and using plastic straws (Zhang and Rawson, 1996). The present vitrification study was carried out using the most permeable embryo stage (1-cell), ultra-rapid cooling method with gold electron microscope grids and N_2 slush (Steponkus *et al.*, 1990; Mazur *et al.*, 1992a), and most penetrating cryoprotectant methanol (Zhang and Rawson, 1996a; Hagedorn *et al.*, 1996). The improved morphological survival following vitrification indicated that the method employed in this study was partially effective. However, no embryos showed viability and poor cryoprotectant permeation and embryo dehydration, and consequently intraembryonic ice formation remain as the biggest problem in vitrification.

7.2.3 Chilling injury in zebrafish embryos

Previous studies on the chilling sensitivity of zebrafish embryos demonstrated that the chilling sensitivity was highly stage-dependent, but there was no published data on whether or not the chilling injury in these fish embryos was related to cooling rate and high development rate of the embryos, which was proposed as one possible mechanism of indirect chilling injury by Mazur *et al.* (1992). As the sensitivity of porcine embryos to lowered temperatures was reported to be related to their high lipid content (Nagashima *et al.*, 1994), the possibility that the chilling sensitivity of zebrafish embryos could be associated with high lipid content in the yolk was also tested. The chilling sensitivity studies indicated that:

(a) the chilling injury in zebrafish embryos was cooling rate related. Later stage (50%-epiboly to 6-somite) embryos that were rapidly cooled and held at -5°C for 1 h demonstrated cold shock injury;
(b) the high chilling sensitivity of zebrafish embryos appeared to be not associated with the high development rate;
(c) both cold shock and indirect chilling injury were yolk related, and partial removal of yolk could reduce both type of chilling injury in zebrafish embryos.

These findings suggest new hope for the application of controlled slow cooling to the cryopreservation of fish embryos. In terms of overcoming chilling injury, yolk-reduced embryos appeared to offer the most likely route for both slow cooling and vitrification.

7.2.4 Temperature of intraembryonic freezing

Experimental evidence suggests a strong correlation between intracellular ice formation and cell injury for many cell types (Mazur, 1984). To maximise survival of zebrafish embryos during a cooling/warming cycle for cryostorage at -196°C, conditions must be established to minimise the possibility of intraembyonic/intracellular ice formation. DSC studies of intraembryonic freezing indicated that:

(a) the temperature of intraembryonic freezing of intact embryos decreased with increasing embryo development;

(b) dechorionated embryos had lower intraembryonic nucleation temperatures than those of intact embryos;
(c) cooling rate appeared to have no effect on the temperatures of intraembryonic freezing;
(d) yolk-reduced embryos exhibited lower intraembryonic nucleation temperatures;
(e) the treatment of 2 M methanol decreased the intraembryonic nucleation temperature of dechorionated 6-somite embryos;
(f) methanol and PG did not depress the intraembryonic nucleation temperature of dechorionated prim-6 and yolk-reduced high-pec embryos;
(g) cryoprotectant (methanol or PG) treatment decreased the intraembryonic nucleation temperature of punctured prim-6 or 6-somite embryos.

These are the first reported intraembryonic nucleation temperatures for fish embryos by DSC, and provided essential information for future studies of the kinetics of subzero chilling injury in zebrafish embryos. The study also provided important information for the design of slow cooling protocols for the cryopreservation of the embryos. DSC is used for the first time to qualitatively determine cryoprotectant penetration by measuring the depression of nucleation temperature by cryoprotectants and reveals that multi-puncturing of the embryos results in a significant cryoprotectant permeation into 6-somite and prim-6 embryos.

7.3 Conclusions

Considerable progress has been made in understanding four key areas that are crucial to the development of methods for mitigating the difficulties confronting the cryopreservation of zebrafish embryos. Table 7.1 summarises the approaches and main achievements of the present study.

7.3.1 Development of methods for reducing yolk

The presence of a large quantity of yolk is considered to be one of the main reasons for the difficulty of achieving fish embryo cryopreservation (Rall, 1993). In the present study, an effective method for reducing the high yolk content in zebrafish embryos without major

Table 7.1 Approaches and achievements of the present study regarding the limiting factors related to the cryopreservation of zebrafish embryos

Limiting factor	Approach	Outcome
Yolk size	Partial removal of yolk	Higher than 50% embryo survival following 50 to 75% yolk removal of post prim-6 stage embryos
High chilling sensitivity	1) Development arrest by anoxia	1) Demonstrated that chilling sensitivity can not be reduced by the arrest of development
	2) Partial removal of yolk	2) Reduction of chilling sensitivity for post prim-6 stage embryos
Low permeability to cryoprotectants	1) Using 1-cell stage embryos, methanol and ultra-rapid cooling	1) Limited improvement in morphological survival, but no viability after vitrification
	2) Multi-punctures of yolk sac	2) Increased cryoprotectant penetration for 6-somite and prim-6 stage embryos

loss of embryo survival has been reported for the first time. Post prim-6 stage embryos appeared to be not complicated by the big yolk size for their cryopreservation since they can well tolerate the damage resulting from the de-yolk procedures, whilst embryos at stage earlier than prim-6 succumb to the partial removal of yolk (Chapter 5).

As the yolk is removed by manually puncturing the yolk sac, the amount of yolk released from the embryo could not be precisely controlled. Embryos also endured severe physical impact during multi-punctures, which was severely injurious to earlier stage (e.g. 6-somite) embryos (Chapter 5). If the de-yolk procedure could be improved by so that it was better controlled and less injurious, it could be used to investigate the effect of partial removal of yolk on earlier stage (pre-prim-6) embryos which have bigger yolk and higher chilling sensitivity. Micro-manipulation could be one way to improve it.

7.3.2 Reduction of chilling sensitivity of zebrafish embryos

High chilling sensitivity of zebrafish embryos precludes the application of controlled slow cooling. This present study reports, for the first time, approaches to reducing the high chilling injury in zebrafish embryos. The chilling sensitivity of the embryos were reduced

after 24 h recovery following the partial removal of their yolk at prim-6 and high-pec stages. However, this approach is not applicable to embryos at stages earlier than prim-6, which are highly sensitive to the partial de-yolk (Chapter 5).

One consideration relating to embryo developmental stage is whether these later stage embryos are appropriate for cryopreservation because they have highly differentiated and also developed epithelia, another permeability barrier, after 24 h recovery period. The successful cryopreservation of invertebrate and insect larvae (Leopold, 1983; Lowrie, 1991; Olive and Wang, 1997) suggests that highly differentiated embryos are not necessarily a problem for cryopreservation. However, the DSC study indicated that the yolk-reduced high-pec embryos were impermeable to both methanol and PG. The advantage of the reduction of chilling sensitivity is therefore undermined by the permeability problems of embryos at this stage.

7.3.3 Increase in cryoprotectant penetration

Previous studies on the permeability of zebrafish embryos suggested there are permeability barriers in the embryos: yolk syncytial layer (Hagedorn *et al.*, 1996) or plasma membrane; (Zhang and Rawson, 1996), but so far little is known about the exact nature of these barriers. In the present study, a new approach to tackling the permeability problem was developed by puncturing the yolk sac. Both 6-somite and prim-6 embryos exhibited increased cryoprotectant penetration after multi-punctures of their yolk (Chapter 6). However, this approach would be ineffective for embryos at later stages like high-pec, which have developed another possible permeability barrier outside the yolk and the yolk only constitutes small part of the embryos.

One concern is to know if these punctured embryos became more sensitive to cryoprotectant. The present study indicated that the punctured 6-somite and yolk-reduced prim-6 embryos can tolerate 1 M methanol for 2 h and 1 M PG for 30 min at room temperature respectively although they appeared to be more sensitive to other cryoprotectants (Chapter 5). The cryoprotectant treatment time required for cryopreservation could be shorter because of the increased cryoprotectant penetration and therefore minimise the toxicity of cryoprotectant. Another possible approach for reducing

the toxicity of cryoprotectants for yolk-reduced embryos is by supplementing the yolk sac with non-toxic or less toxic cryoprotectants such as trehalose or other sugars.

7.3.4 Potential protocol for future zebrafish cryopreservation

Considering embryo chilling sensitivity and their intraembryonic nucleation temperatures, the combination of the use of yolk-reduced embryos at high-pec stage and controlled slow cooling may increase the chance of successful cryopreservation of zebrafish embryos because of the significant reduction in both chilling injury (Chapter 5) and intraembryonic nucleation temperatures (Chapter 6) in the yolk-reduced embryos. However further studies on kinetics of subzero chilling injury are needed to decide whether the chilling injury could be reduced sufficiently to allow slow cooling approach for these embryos. Moreover, the yolk-reduced embryos, which need 24 h recovery to reduce their chilling sensitivity, appeared to be impermeable to cryoprotectants (Chapter 6) probably because of the development of the new melanised epithelia. As the yolk constitutes a very small fraction of the yolk-reduced embryos at high-pec stage, increasing the cryoprotectant permeation into the yolk-reduced high-pec embryos by multi-punctures of the yolk is ineffective, and new methods for increasing their permeability still need to be developed if this stage is to be considered for cryopreservation.

From the cryoprotectant penetration point of view, punctured 6-somite and prim-6 embryos appear to be the best candidates for future cryopreservation because of the increased cryoprotectant permeation into these punctured embryos (Chapter 6). Comparing these two embryo stages, 6-somite stage is less preferable as embryos at this stage are very sensitive to yolk-removal (Chapter 5) and could not benefit from the reduction of yolk size and also chilling sensitivity by the partial removal of yolk although dechorionated 6-somite embryos appeared to be more permeable to cryoprotectant methanol (Chapter 6). Because prim-6 embryos can survive reasonably well (> 50%) after partial yolk removal and the treatment of 1 or 2 M methanol (Chapter 5), the combined advantage of the reduction of embryo size by yolk removal (Chapter 5) and the increased cryoprotectant permeation into the embryos by multi-punctures (Chapter 6) could greatly benefit the vitrification approach applied to the cryopreservation of the embryos. As vitrification can overcome the chilling sensitivity, it will be unnecessary for the yolk-reduced prim-6 embryos to have a long period (24 h) of recovery time to reduce their chilling sensitivity. Methanol is most likely to be the

main cryoprotectant for the future vitrification of yolk-reduced prim-6 embryos since it's toxicity to the embryos is relatively low (Chapter 3, 5). The ultra-rapid cooling method achieved by mounting a single embryo on a gold electron microscope grid and plunging it into LN_2 or N_2 slush could also be considered in the future because of its positive effect on the vitrification of intact embryos (Chapter 3).

7.4 Future work

As vitrification is preferable for the cryopreservation of yolk-reduced embryos at prim-6 stage, the first important area for future work is the toxicity study of methanol based vitrification solution to the embryos. Measures should be taken to reduce the potential toxicity of vitrification solution since the yolk-reduced embryos become more sensitive to cryoprotectants (Chapter 5). The mixture of several different components and the inclusion of non-permeating cryoprotectants could be helpful to formulate a less toxic vitrification solution (See section 1.2.5.2). Supplementation of yolk sac with sugars and/or anti-freeze glycoproteins during yolk-removal to reduce the toxicity of vitrification solution and also prevent devitrification during warming could also be beneficial.

The second area for future investigation is the study of kinetics of subzero chilling injury in yolk-reduced high-pec embryos so as to determine whether partial removal of yolk could ameliorate the chilling sensitivity at subzero temperatures sufficiently to allow the application of controlled slow cooling for their cryopreservtion. The present DSC study indicated that the yolk-reduced embryos supercooled to below -27°C, the kinetics of chilling injury can be determined down to this temperature. At the same time, new approaches also need to be explored to increase the permeability of embryos at this stage since they have developed melanised epithelia, a new possible permeability barrier. It is believed that this epithelia barrier in mosquito embryos is associated with cross-linking of tyrosyl residues in the plasma (vitelline) membrane, which is driven by phenoloxidase (Clements, 1992). If this is also true with zebrafish embryos, one possible approach to overcoming this problem is to impede the cross-linking by using phloroglucinol (Valencia *et al.*, 1997), which is a known inhibitor of phenoloxidase reaction. Other methods including chemical treatment using sodium hypochlorite and enzymatic digestion using pronase E, which have recently been

reported to increase the cryoprotectant permeability of turbot embryos (Cabrita *et al.*, 1999), also need to be investigated in zebrafish embryos.

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APPENDIX: Publications

The following co-authored papers were produced during the course of the research project:

Liu, X.-H., Zhang T., and Rawson, D. M. (1997) A preliminary study on vitrification of intact zebrafish (*Brachydanio rerio*) embryos using methanol. *Cryobiology* **35**, 372. [abstract]

Liu, X.-H., Zhang T., and Rawson, D. M (1998) Feasibility of vitrification of zebrafish *(danio rerio)* embryos using methanol. *Cryo-letters* **19**, 309-318.

Liu, X.-H., Zhang T., and Rawson, D. M. (1999) Effect of partial removal of yolk on the survival and chilling sensitivity of zebrafish (*danio rerio*) embryos. *Cryo-letters* **20**, 136. [abstract]

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